REVIEW / SYNTHÈSE

Phage display: applicati ns, innovati ns, and issues in phage and host biology

Dan R. Wilson and B. Brett Finlay

Abstract: In the 7 years since the first publications describing phage-displayed peptide libraries, phage display has been successfully employed in a variety of research. Innovations in vector design and methods to identify target clones account for much of this success. At the same time, not all ventures have been entirely successful and it appears that phage and host biology play important roles in this. A key issue concerns the role played by a displayed peptide or protein in its successful expression and incorporation into virions. While few studies have examined these issues specifically in context of phage display, the literature as a whole provides insight. Accordingly, we review phage biology, relevant aspects of host biology, and phage display applications with the goals of illustrating (*i*) relevant aspects of the interplay between phage–host biology and successful phage display and (*ii*) the limitations and considerable potential of this important technology.

Key words: bacteriophage M13, phage display, pIII, pVIII, expression libraries.

Résumé : Au cours des 7 années qui ont suivi les premières publications décrivant des banques de peptides exposés à la surface de phages, cet affichage d'un phage a été utilisé avec succès dans divers types de recherche. Des innovations dans la mise au point de vecteurs et de méthodes d'identification de clones-cibles expliquent une bonne partie de ce succès. Toutes les initiatives n'ont cependant pas été couronnées de succès car la biologie du phage et de son hôte jouerait un rôle important. Un développement crucial consiste à élucider le rôle joué par une protéine ou un peptide exposé à la surface du phage dans son incorporation et son expression efficace dans les virions. Même si peu d'études ont porté sur les applications de l'affichage d'un phage, l'ensemble de la littérature propose des suggestions intéressantes. Dans cette perspective, nous passons en revue la biologie des phages, les aspects pertinents de la biologie de l'hôte et les applications de l'affichage du phage en vue d'identifier les points importants de l'interaction hôte–phage et de la réussite de l'affichage du phage. Nous voulons aussi illustrer le potentiel considérable et aussi les limites de cette technologie importante.

Mots clés : bactériophage M13, affichage d'un bactériophage, pIII, pVIII, banques d'expression.
[Traduit par la rédaction]

Received August 11, 1997. Revision received November 13, 1997. Accepted January 13, 1998.

D.R. Wilson. Department of Microbiology and Immunology and Biotechnology Laboratory, University of British Columbia, Vancouver, BC V6T 1W5, Canada.
B.B. Finlay.[1] Department of Microbiology and Immunology, Biotechnology Laboratory, and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1W5, Canada.

[1] Author to whom all correspondence should be sent at the following address: Biotechnology Laboratory, Room 237, Wesbrook Building, 6174 University Boulevard, University of British Columbia, Vancouver, BC V6T 1W5, Canada.

EXHIBIT B

Introduction

The publication in 1990 of three studies describing peptide display on the surface of a filamentous bacteriophage brought to the attention of many scientists a simple and powerful approach to identifying or optimizing ligands for antibodies and other biomolecules. In model studies, three groups of researchers had constructed vast libraries of random sequence peptides fused to the N-terminus of a minor coat protein (pIII) of a filamentous phage (Fig. 1) and by simple affinity-selection methods, identified novel sequences that bound specifically to cognate antibodies (Scott and Smith 1990; Cwirla et al. 1990) or streptavidin (Devlin et al. 1990).

Several aspects of phage biology made these studies possible and the concept powerful. Because large numbers of virions occupy a small volume ($>>10^{12}$ particles·mL^{-1}), construction of libraries of the required diversity ($>10^7$ unique clones; by cloning degenerate oligonucleotides into gIII;[2] Fig. 1C) and their subsequent employment was achievable. Display of peptides in the simple physicochemical context of a structurally simple virion (Fig. 1B) allowed for successful enrichment with minimal background. Importantly, affinity-selection of a target peptide served to clone its encoding DNA (Fig. 1D) because the peptide and DNA were physically linked.

Phage display soon found a diversity of applications. Among early studies were pIII display of a properly folded human growth hormone (Bass et al. 1990) and of single-chain Fv antibodies (McCafferty et al. 1990), as well as both pIII and pVIII (the major coat protein) display of Fab fragments (Barbas et al. 1991; Kang et al. 1991). Affinity-selection other than with antibodies was soon demonstrated in a study called "directed evolution of a protein," which employed a library of variants of bovine pancreatic trypsin inhibitor (BPTI) to identify a peptide possessing $>10^6$-fold greater affinity for human neutrophil elastase than the starting peptide (Roberts et al. 1992*a*).

The value of phage display is reflected in the large number of published reviews. These include general overviews (Wells and Lowman 1992; Barbas 1993; Smith and Scott 1993; Clackson and Wells 1994; Bradbury and Cattaneo 1995; Perham et al. 1995; Smith and Petrenko 1997) and reviews of random peptide libraries (Scott 1992; Lane and Stephen 1993; Scott and Craig 1994; Cortese et al. 1994, 1995, 1996; Burritt et al. 1996) and display of proteins (Dunn 1996), as well as more focussed reviews concerning affinity-maturation themes (O'Neil and Hoess 1995), display of constrained peptides (Ladner 1995), antibody display (e.g., Marks et al. 1992; Winter et al. 1994), and the use of zinc finger libraries (Choo and Klug 1995).

Notwithstanding the successful employment of phage display in a variety of research problems, not all applications have been entirely successful and it appears that phage and host biology play important roles in this. Expectedly, as with protein expression generally, a key issue concerns the ways in which a displayed peptide or protein influences its ability to be expressed and incorporated into virions. Although few studies have examined these issues specifically in context of phage display, the literature as a whole provides insight. Accordingly, we review relevant aspects of phage and host biology and phage display applications with the goals of (*i*) shedding light on the ways in which phage and host biology can interfere with phage display and (*ii*) illustrating both the limitations and considerable potential of this important technology.

Ff phage biology

Overview

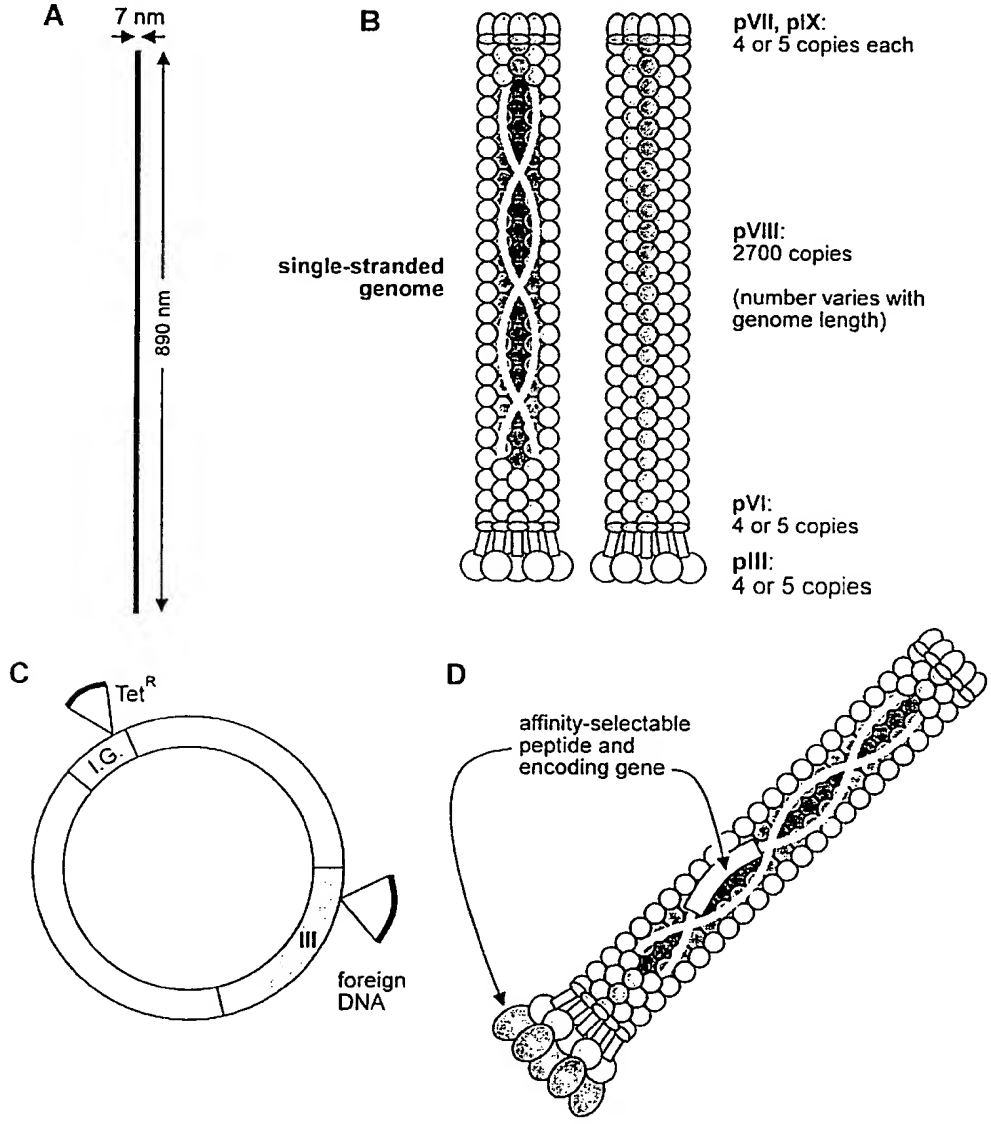
M13, f1, and fd (reviewed[3] in Rasched and Oberer 1986; Model and Russel 1988; Russel 1991, 1995) are collectively called the Ff phage because of their filamentous appearance and almost total dependence (Russel et al. 1988) on the F-pilus for infection of their *Escherichia coli* host. The long (900 nm) and narrow (7 nm) Ff virion (Fig. 1A) consists of a single-stranded (ss) DNA genome packaged in a tube comprised of 2700 copies of the major coat protein pVIII and closed at the ends by four or five copies of each of four species of minor coat proteins, including pIII (Fig. 1B). Unlike lytic phage, which are released by cell lysis after assembly in the host cell cytoplasm, Ff phage are continuously extruded through the host cell envelope in a process that couples assembly with export.

Infection begins when pIII adsorbs to the tip of a host cell

[2] Several conventions exist for identifying Ff phage genes and their products. Commonly, phage genes are identified with Roman numerals (e.g., gene III, gene VIII), while their encoded proteins are identified with Roman numerals prefixed with the letter p (e.g., pIII, pVIII). For clarity and convenience, we identify genes and their products with the prefixes g and p, respectively (e.g., gIII, gVIII; pIII, pVIII).

[3] Except where noted, material presented in this section is derived from the cited reviews.

Fig. 1. Wild-type Ff phage and multivalent pIII phage display. (A and B) Wild-type filamentous phage that infect F-piliated *E. coli* (Ff phage). (A) Ff phage are narrow flexible rods of the indicated dimensions. (B) Cartoon illustrates inherent simplicity of virion structure: a single-stranded circular genome is surrounded by ~2700 copies of the major coat protein pVIII and 4 or 5 copies of each of four species of minor coat proteins, including pIII, which binds to a host cell F-pilus. For a model derived from X-ray fibre diffraction and other data, the interested reader is directed to Marvin et al. (1994). (C) A number of multivalent pIII phage display vectors are derivatives of fd-tet (Zacher et al. 1980) and accordingly possess a tetracycline-resistance gene (Tet^R) inserted into the intergenic (I.G.) region. (C and D) Inserting foreign DNA into gIII at a point corresponding to the pIII N-terminal signal peptidase cleavage site results in the display of a foreign peptide as an N-terminal fusion to each mature pIII molecule. (D) As described in the text, a key feature of phage display libraries is that an affinity-selectable target peptide and its encoding DNA are packaged together.



F-pilus. After the virion is brought to the cell surface and the ss genome (or (+) strand) is delivered to the cytoplasm, host polymerases employ the (+) strand as a template for synthesis of a complementary (–) strand, yielding a double-stranded (or replicative form (RF)) phage genome. Synthesis of the (–) strand begins at *ori*(–), one of several sites within the intergenic region. Because of the double-stranded nature of two adjacent stem loops, *ori*(–) resembles the –35 and –10 regions of RNA promoters; the resemblance is important for primer synthesis by RNA polymerase (Higashitani et al. 1997). Surprisingly, mutants with defects in *ori*(–) are viable, including M13 mutants with deletions in *ori*(–) (Kim et al. 1981) and fd-tet, a vector constructed by splicing a tetracycline-resistance gene into *ori*(–) (Zacher et al. 1980; Smith 1988) at a position that interrupts one of the stem loops. Initiation of (–) strand replication is delayed in these mutants and proceeds slowly by an unidentified mechanism (Kim et al. 1981; Smith 1988; Higashitani et al. 1992).

Following (–) strand synthesis, host-mediated processes lead to production of coat (pIII, pVI, pVII, pVIII, pIX), assembly and export (pI and pIV), and phage replication (pII, pV, pX) proteins, the levels of which are determined mainly by a

conceptually simple mechanism. Thus, the genome is organized into two coding regions, one for proteins required in large quantities (e.g., pVIII) and possessing a strong initial promoter and the other for proteins required in fewer numbers (e.g., pIII) and possessing a less efficient initial promoter. Multiple promoters within each region lead to more frequent transcription of 3′ versus 5′ genes. Host-mediated degradation of mRNA transcripts from the 5′ end magnifies this cascade effect, so that a finely tuned balance of synthesis of phage proteins is achieved with little apparent feedback control (but see Fulford and Model (1988*a*, 1988*b*) for regulation of (–) strand synthesis by pII, pX, and pV). Prima facie, this balance would appear to be sensitive to perturbation.

Phage replication is initiated by pII, which nicks the (+) strand at *ori*(+) in the intergenic region, allowing the freed 3′ end to serve as a primer for rolling circle replication on the (–) strand template. As each nascent (+) strand is completed, pII ligates the molecular ends to form a closed circle. Early in infection, new (+) strands serve as templates for additional RF molecules as described earlier, while later in infection, ss-to-RF conversion is arrested as nascent (+) strands become sequestered by increasing levels of the ss DNA binding protein pV.

Virion assembly and export require the host cell membranes. Following synthesis, all five coat proteins are inserted into the inner membrane (Endemann and Model 1995). Although pIII and pVIII are synthesized with N-terminal signal peptides that are cleaved upon membrane insertion, they remain anchored in the membrane by C-terminal hydrophobic regions with their N-termini in the periplasm. Whereas membrane insertion of pIII may be Sec dependant because of its relatively large size (for size as a determinant of Sec dependence, see Pugsley (1993)) and recombinant pIII appears to require Sec functions (Peters et al. 1994), pVIII insertion is regarded as Sec independent (Pugsley 1993; Kuhn 1995; Murphy and Beckwith 1996). In current models, pI and pIV form a gated multimeric export channel spanning the inner and outer membranes. Initiation of assembly and export involves interactions among a packaging signal within the intergenic region, the pI–pIV complex, host thioredoxin, and minor coat proteins pVII and pIX. In a process involving ATP hydrolysis and proton motive force (Feng et al. 1997), nascent virions are extruded through the export channel with the concomitant replacement of pV by pVIII. Addition of pVI and pIII to the end of an extruding virion completes its assembly.

pIII

pIII is required not only for F-pilus adsorption but also for terminating virion assembly and stabilizing the viral particle. Delays in supplying pIII lead to production of multilength viral particles (polyphage) containing two or more unit-length phage genomes (Rasched and Oberer 1986; Model and Russel 1988); even wild-type Ff phage routinely produce about 5% polyphage. The knob-on-stem appearance of pIII in electron micrographs reflects its two functional domains. The N-terminal two-thirds of the molecule mediates virion adsorption while the C-terminal one-third functions in virion assembly and structure (Armstrong et al. 1981; Crissman and Smith 1984; Model and Russel 1988). Thus mutants producing pIII that lacks a substantial portion of the N-terminal domain produce normal numbers of noninfectious unit-length virions (monophage; Crissman and Smith 1984), but mutants unable to produce at least the C-terminal portion of pIII are host lethal or (in an fd-tet background, described later) produce relatively less stable noninfectious polyphage (Pratt et al. 1966; Crissman and Smith 1984).

Many of the membrane-associated phenotypes exhibited by Ff phage infected cells, such as increased detergent sensitivity and leakage of periplasmic proteins, are mediated by pIII (Nelson et al. 1981; Boeke et al. 1982; Rampf et al. 1991).

Early phage display

The first phage display library

The first phage display library (PDL) was constructed by Smith (1985), who cloned *Eco*RI gene fragments into gIII of phage f1, creating a library of virions displaying peptides between the pIII N- and C-terminal domains. Although identification of a clone reactive with α-EcoRI antibodies demonstrated the considerable promise of this novel approach, Smith noted several problems related to phage biology. The identified clone produced small, barely detectable plaques and increased numbers of polyphage. Infectivity was reduced 25-fold and loss or alteration of the *Eco*RI insert conferred a marked selective advantage during virion propagation.

fd-tet

Ff mutations that block phage assembly while allowing DNA replication to continue are normally lethal to the host, possibly because of intracellular accumulation of phage DNA and gene products (Pratt et al. 1966; Smith 1988; Smith and Scott 1993). Because of its *ori*(–) defect, however, the RF copy number of fd-tet is reduced about 13-fold and otherwise lethal (in a wild-type background) morphogenetically defective mutants of fd-tet are thus viable (Crissman and Smith 1984; Smith 1988; Smith and Scott 1993). Parmley and Smith (1988) exploited this property of fd-tet in designing frameshift vectors, described later.

Compared to wild-type Ff, fd-tet plaques are extremely small and turbid; virion production is reduced fourfold, and yields of RF and ss DNA are reduced more than 10-fold (Smith 1988). M13 *ori*(–) mutants have similar plaque phenotypes and reduced virion yields (Kim et al. 1981). Infectivity of fd-tet (infectious units/physical particle) is reduced to as little as 2.5% of that of wild-type Ff (Smith 1988), although infectivity of an fd-tet derived vector was found to be 50% of wild-type levels (Parmley and Smith 1988).

SOS response induction

Relatively recently, M13 mutants with *ori*(–) defects have been shown to induce the *E. coli* SOS response (Higashitani et al. 1992, 1995), presumably because of delayed (–) strand synthesis or related events that expose the cell to ss DNA, the apparent SOS system inducer (Walker 1996). The phenotypic similarities of M13 *ori*(–) mutants and fd-tet suggest the latter may also induce the SOS response. Generally, SOS induction leads to increased spontaneous mutagenesis (Hutchinson 1996), such as in certain base substitution mutations (Miller and Low 1984; Yatagai et al. 1991*a*, 1991*b*), as well as increased induction of *recA* and other recombination-related genes (Lloyd and Low 1996; Walker 1996) and a consequent

increased frequency of homologous recombination (Abbot 1985; Dolzani et al. 1991; Mudgett 1991). Given that many phage display vectors have been derived from fd-tet (Smith and Scott 1993; Bonnycastle et al. 1996), SOS response induction by this vector merits investigation.

The first biopanning

Continuing Smith's work, Parmley and Smith (1988) showed that virions displaying peptides of interest could be affinity-selected (or biopanned) from a mock library containing a huge (10^8-fold) excess of virions lacking the target peptide. Several improvements, reflecting a consideration of phage biology, were made in vector design. Displayed peptides were moved from the earlier mid-pIII site to the N-terminus of mature pIII, allowing for greater conformational freedom and reducing potential interference with the adsorption function of pIII. As well, fd-tet (versus wild-type f1) was employed as a parent for new vectors, with the hope that the reduced gene expression and virion production of fd-tet (Smith 1988) might diminish the host toxicity of some difficult to export peptides (Smith and Scott 1993). Finally, knowing that pIII-deficient mutants of fd-tet were viable, Parmley and Smith (1988) developed a frameshift vector (fUSE1) in which a frameshift at the gIII cloning site prevented production of pIII and thus of infectious virions, unless a foreign insert restored the gIII reading frame. Here the goal was to allow construction of libraries in which each infectious virion was a recombinant, a clear advantage in eliminating possible overgrowth of nonrecombinants during library propagation and in assessing cloning success and the completeness of a library.

Some problems remained, particularly with fUSE1 recombinants bearing larger inserts. Thus, 54- and 71-bp inserts reduced infectivity by 50%, and a 335-bp insert reduced infectivity 20-fold. Importantly, a 20-bp insert had no apparent effect on infectivity.

The first random peptide libraries

Three pioneering random peptide library (RPL) applications (Cwirla et al. 1990; Devlin et al. 1990; Scott and Smith 1990) validated the proposal of Parmley and Smith (1988) that large libraries of phage-displayed peptides could be used to identify epitopes, mimotopes (Geysen et al. 1987), or other mimetic peptides. To construct their $>10^7$-member hexapeptide RPL, Scott and Smith (1990) designed both their vector (fUSE5) and inserts in a way that reflected a consideration of phage and host biology. To minimize problems in signal peptidase processing of recombinant pIII, the first two residues following the cleavage site were retained (with Asp substituting for the normal Glu in Ala–Glu) in their clones; these were followed by a random hexapeptide flanked by Gly–Ala-rich linker sequences. Other features of fUSE5, including a gIII frameshift, served to exclude nonrecombinants from their library. Cwirla et al. (1990) also used an fd-tet derived frameshift vector to construct their hexapeptide RPL; here, however, random peptides were inserted immediately following the signal peptide cleavage site.

Histobiology

From the notable successes of these RPL studies, it seemed evident that phage–host biology did not interfere with the display of small pIII-displayed peptides, at least in ways that were of practical consequence. The previously reviewed successes with display of proteins, such as human growth hormone, bovine pancreatic trypsin inhibitor variants, and antibodies, tended to support this idea. Yet, as expected from the earliest studies and anticipated by Parmley and Smith (1988), a review of the issues involved should predict that many applications may not succeed for the reason that, as with recombinant technology generally, Ff phage display requires host cell processes for both synthesis and export of recombinant pIII or pVIII molecules.

Intuitively, we expect diverse proteins to make varying demands on the export appartatus and indeed, a body of experimental data (Pugsley 1993) suggest that proteins vary in their requirements (e.g., for specific components of the Sec apparatus; for membrane potential) for efficient export. Accordingly, members of a compositionally diverse PDL may be subjected to different constraints, leading to varying degrees of success in display of member peptides.

Signal peptides and the N-terminal mature region

Importantly, phage display involves fusions to or insertions near the N-termini of mature pIII or pVIII, a region which in proteins is generally believed to play a role in export. Indeed, virtually all RPLs display peptides within a postulated N-terminal "critical 30-residue export initiation domain" (Andersson and von Heijne 1991; Nielsen et al. 1997; also see Summers and Knowles 1989).

Both pIII and pVIII preproteins possess N-terminal signal peptides that are cleaved upon membrane insertion and much of our understanding of this process has derived from studies employing pVIII (Model and Russel 1988; Pugsley 1993). Both processing by signal peptidase and subsequent virion production are affected in some pVIII mutants possessing residue substitutions within the N-terminal mature region. For example, pVIII preprotein cleavage is reduced by an Ala→Cys substitution at position +1 (viz., immediately following the cleavage position) and abolished by Ala→Pro or Ala→Thr substitution. A Glu→Leu or Glu→Tyr substitution at position +2 retards pVIII processing and, in the case of a Glu→Leu substitution, affects the kinetics of virion production (Boeke et al. 1980; Russel and Model 1981). Although pVIII tolerates a number of substitutions at positions +1 to +5 (Iannolo et al. 1995; Williams et al. 1995), certain substitutions (most notably by Tyr) can reduce virion titers by $>10^{10}$-fold (Iannolo et al. 1995).

Early difficulties (e.g., Greenwood et al. 1991; Felici et al. 1991; see also Perham et al. 1995) in pVIII display of peptides larger than six amino acids raised the possibility of a size or other restriction related to virion assembly (e.g., steric interference in pVIII–pVIII interactions) or export through the pI–pIV channel and there is evidence (Iannolo et al. 1995; Williams et al. 1995) that such restrictions exist. On the other hand, X-ray diffraction studies and model building (Malik et al. 1996) have indicated that pVIII is able to display larger structures than accomplished thus far in vivo. Importantly, related work (Malik et al. 1996) found that the success of pVIII display correlated better with efficiency of preprotein processing by signal peptidase than with the sizes of displayed peptides.

Study of the N-terminal mature region of recombinant pIII

is limited to a single report (Peters et al. 1994) where it was found that (*i*) 5% of clones in a hexapeptide RPL yielded 10- to 10^5-fold fewer virions than a parental (fd-tet) phage, (*ii*) positively charged (Arg) residues within the mature pIII N-terminal decamer of a second PDL reduced virion titers in a dose-related manner from ~10^{10} virions·mL^{-1} to as little as 10^3 virions·mL^{-1}, and (*iii*) *prlA* (*secY*) suppressors could restore virion production of most defective clones 10^6- to 10^7-fold. Charged residues in the N-terminal mature region have been shown to hinder translocation of other recombinant proteins (e.g., Li et al. 1988; Andersson and von Heijne 1991; Yamane and Mizushima 1988; reviewed in Boyd and Beckwith 1990). It has been suggested that (*i*) these disrupt a net dipole formed around a signal peptide's hydrophobic domain and required for translocation (Boyd and Beckwith 1990; Izard and Kendall 1994) and (*ii*) clusters of these residues may, by conforming to the positive inside rule (von Heijne 1994; see also Yamane and Mizushima 1988) derived from study of membrane proteins, block membrane translocation of the signal peptide C-terminal domain.

Surveys of prokaryotic signal peptides and flanking sequences (Nielsen et al. 1997; von Heijne 1986) have revealed a marked distribution of amino acids around the signal peptidase cleavage site, including preprotein (–1, –2, ...) and mature protein (+1, +2, ...) positions. For example, Pro is relatively common in positions –6 to –4 and +2 to +6 but rare[4] in positions –3 to +1 (Nielsen et al. 1997). Ala is the most common (mol% = 38.3) residue at +1, while Asp (16.2%) and Glu (15.0%) predominate at +2. Notably, these (Ala–Asp or Ala–Glu) residue pairs initiate mature pIII of wild-type Ff phage and, by design, of recombinants in the RPLs of Scott and Smith (1990) and Devlin et al. (1990) and of several phage display vectors (Burritt et al. 1996). In contrast, foreign peptides are displayed at, rather than a few residues away from, the +1 position of pIII in the RPL of Cwirla et al. (1990). The evidence above suggests that this latter approach may be problematic.

We examined this issue theoretically following experimental work that employed phage libraries displaying peptides derived from the *Bordetella pertussis* filamentous hemagglutinin gene (Wilson 1997) by using the neural networks of Nielsen et al. (1997) to predict signal peptide recognition and cleavage expected from display of the 30 target clones we identified. Predictions were based on use of (*i*) the pIII display vectors we employed, in which peptides follow the N-terminal spacer sequences Ala–Asp–Gly–Ala–Gly–Ala (vector fDRW70, similar to fUSE5 of Scott and Smith (1990)) and Ala–Asp–Gly–Pro (fDRW8*nn* vectors) and (*ii*) a hypothetical pIII vector that displays a peptide without a spacer, viz., at the N-terminus. High cleavage site scores were reported for all fDRW70 constructs. Most fDRW8*nn* constructs scored below a default cutoff value (0.49), but only marginally so (≥0.43). In contrast, half of the constructs in the third hypothetical vector yielded scores less than 0.3, with some as low as 0.15; moreover, most constructs were predicted to have altered cleavage sites.

Because pIII and pVIII preproteins possess N-terminal (signal peptide) and C-terminal (membrane anchor) hydrophobic domains, foreign inserts that hinder signal peptide cleavage could effectively convert these preproteins to polytopic membrane proteins. Given the crude regulation of phage gene expression and general lack of feedback control, elevated membrane accumulation of pIII (which at normal levels mediates membrane effects) or pVIII would be expected, to the detriment of the host. Consistent with this, mutants in which pVIII synthesis continues while phage morphogensis is blocked are host lethal and mediate a variety of membrane effects (Russel and Model 1981); these include mutants blocked in signal peptidase processing (Rasched and Oberer 1986).

Other aspects of membrane insertion–translocation

The amino acid sequence of a protein can influence more than signal peptidase activity. For example, the normally Sec-independent membrane insertion of pVIII became Sec dependent when the size of periplasmic loop (transiently formed between the signal peptide's hydrophobic core and the C-terminal membrane anchor) was increased and in a related way Sec dependence of a model membrane protein increased concomitantly with the size of its periplasmic loop (von Heijne 1994). It has been postulated that only relatively short polypeptides are candidates for Sec-independent membrane translocation and their small size may actually preclude productive interaction with some Sec components (Pugsley 1993). In this context, Pugsley (1993) has questioned whether a protein can be designed to be both too long to be Sec independent and too short to be Sec dependent. This intriguing idea suggests that certain intermediate-sized pVIII recombinants may prove to be intrinsically incompetent for export.

Both negatively and positively charged residues can markedly influence membrane translocation (von Heijne 1994; Cao et al. 1995). For example, increasing the negative charge of the periplasmic loop in pVIII and leader peptidase constructs resulted in increased dependence on the electrochemical membrane potential or the Sec apparatus for translocation (Cao et al. 1995). Also, inserting four or five Asp and Glu residues at the beginning or middle of the periplasmic loop of pVIII inhibited membrane insertion or leader peptidase processing to as little as 18% of wild-type levels (Cao et al. 1995).

These findings may account for observations (Grihalde et al. 1995) concerning a 30-residue RPL in which variable residues followed the N-terminal epitope tag DYKDDDDK and four other residues (AETA), the epitope tag having been included to allow assessment of N-terminal proteolysis of recombinant pIII. That only 5–10% of virions displayed an intact tag and infectivity of pooled library virions was only 0.4% are consistent with problems in translocation or processing of the charge-rich sequence. In support of this, the neural networks mentioned earlier (Nielsen et al. 1997) predict that a pIII preprotein sequence constructed with the DYKDDDDK epitope tag lacks a signal peptide.[5]

[4] Calculated mol% values are derived from data employed in the study by Nielsen et al. (1997) and provided by the authors via their FTP server at ftp://virus.cbs.dtu.dk/pub/signalp.

[5] The prediciton was made using the WWW server (http://www.cbs.dtu.dk/) of the Center for Biological Sequence Analysis and the preprotein sequence VKKLLFAIPLVVPF-YSHS–DYKDDDDKAETA, where – indicates the cleavage site in wild-type pIII. The sequence yielded a cleavage site score below (66% of) the default cutoff value for predicting cleavage.

Genomic libraries, membrane anchors, and secondary signal sequences

Genomic PDLs may pose related problems, for a large fraction (20%) of chromosomal sequences can act as signal peptides (Kaiser et al. 1987). Given similarities among signal peptides, signal anchors, and membrane anchors (MacIntyre and Henning 1990; Nielsen et al. 1997) and that requirements for transmembrane segments include only minimal length (12 residues) and hydrophobicity (Pugsley 1993), then a significant fraction of genomic PDL clones may contain motifs that hinder translocation, anchor pIII, or pVIII in the membrane or act as secondary signal peptides (MacIntyre and Henning 1990), yielding unexpected cleavage.

Codon usage and context

Codon usage and translational context in *E. coli* (reviewed briefly in Smith and Smith 1996; Berg and Silva 1997) may adversely affect synthesis of recombinant pVIII (~2700 copies required/virion) and pIII (less so; 4–5 copies/virion), particularly for genomic PDLs derived from genomes with G+C contents substantially different from that of *E. coli*. Codon usage within the *B. pertussis fhaB* gene provides an example with which we are familiar (Wilson 1997). Analysis of 196 *fhaB* Arg codons shows that the most common Arg codon in *E. coli* (CGU) tends to be avoided in *fhaB*, and that *fhaB* employs other Arg codons that are rare in *E. coli* (Dong et al. 1996). These include 15 occurrences of AGG, found to adversely affect recombinant gene expression (Kane 1995). *Escherichia coli* avoids not only the AGG codon but also the AG-G triplet (- denotes a boundary between codons) for reasons possibly related to translation (Smith and Smith 1996; Berg and Silva 1997). Notably, AG-G accounts for less than 4% of a data set of 742 *E. coli* tetranucleotides (Smith and Smith 1996) but 38% of our *fhaB* data set.

Molecular chaperones and export targeting

Nascent and completed proteins interact with a variety of molecules with roles in folding and export, including trigger factor, DnaJ/DnaK and GroEl/GroES, as well as SecB and other Sec components (Pugsley 1993; Hartl 1996; Hesterkamp and Bukau 1996; Mayhew and Hartl 1996; Murphy and Beckwith 1996). Importantly, the attachment of a signal peptide to a normally cytoplasmic protein does not necessarily ensure its export (e.g., Summers and Knowles 1989; Andersson and von Heijne 1991). Rather, a protein may also require certain motifs (patterns of hydrophobicity, positively charged and flexible polypeptide segments) that allow maintenance by molecular chaperones of a partially unfolded export-competent state (MacIntyre and Henning 1990; Pugsley 1993; Driessen 1994; Mayhew and Hartl 1996; Murphy and Beckwith 1996).

Although chaperone-assisted phage display of antibodies (Lah et al. 1994; Söderlind et al. 1995) and recombinant protein export generally (Wall and Pluckthun 1995) have been explored (with mixed results), little is understood regarding the influence of molecular chaperones on the export of recombinant pIII and pVIII. Possibly, fusion of a small peptide to the relatively large pIII (424 residues) has little impact on export competence, whereas fusion of a large normally cytoplasmic protein to the smaller pVIII (50 residues) may pose greater difficulties.

Proteases

Protein degradation (Miller 1996) is a highly active compo nent of *E. coli* metabolism, employed to both regulate leve of functional proteins and eliminate damaged proteins. In ge eral terms, certain alterations tend to increase the rate of de radation of otherwise stable proteins. These include t production of longer or shorter versions of a normal protein amino acid substitutions that lead to exposure of elements th in the native protein are inaccessible. It has been suggested th since proteins and proteases have coevolved, protein evolutic is constrained by a proteolytic machinery effectively designe to recognize nonnative elements. In this context, recombina pIII and pVIII may be candidates for degradation.

As illustrated by later examples, there is evidence that pr tein degradation is an issue in phage display. Yet its impo tance may be obscured by the not uncommon practice inferring the sequence of a displayed peptide from that of i encoding gIII or gVIII insert without corresponding asses ment of the fusion products, such as by SDS–PAGE, Weste blotting, and N-terminal sequencing (as illustrated in Hart al. 1994).

Impact of phage and host biology on phage display

Although problems concerning the quantity and quality of e ported proteins can (or must, out of practical necessity) b ignored in some applications of recombinant technology, the matters assume greater importance with phage display, for t utility of a PDL will often derive from unbiased reliable di play of all encoded peptides. The sections above describe general terms the ways in which phage and host biology ma interfere with phage display. The following sections, in r viewing applications of phage display, provide a more specif sense of the degree to which such interference is an issue ar the extent to which it has been examined.

Applications and innovations in phag display

Random peptide libraries

Library diversity and bias

RPLs displaying peptides of 6–38 amino acids have been d scribed. Two common concerns in library construction are i diversity or completeness and bias. RPLs are generally co structed with only 32 codons (e.g., of the form NNK or NN where K = G or T and S = G or C) to reduce a bias inherent the genetic code from a maximum amino acid molar ratio 6:1 (e.g., Arg versus Met) to 3:1. A hexapeptide library di playing all possible hexapeptides (a complete library) th requires 32^6 (= 10^9) unique clones and, assuming a practic upper limit of ~10^9–10^{10} (Smith 1993), libraries of peptid longer than seven residues accordingly risk being incomplet Nevertheless, libraries of relatively long peptides may ha advantages. For example, assuming that a hexapeptide RPL adequate for a given application, a 20-residue library may i crease the effective library diversity, for each 20-residue pe tide contains 15 hexapeptides in different flanking sequenc (possibly structural) contexts.

Whether a library needs to be complete depends, in a give application, on the contributions to binding of individual res

dues within the target peptide. If all residues contribute equally, then assured isolation of the single most tightly binding clone will require that the library contain all possible clones. Where only a few residues contribute to binding, completeness matters less; what remains important in this case is that there be little or no position-specific bias, such as against certain residues in the residues flanking the pIII signal peptidase site.

That completeness is not always essential can be illustrated by two examples of results obtained from a library of limited diversity. Thus, although the library of Devlin et al. (1990) displayed only $1/10^9$ of the 20^{15} possible 15-residue peptides, it was successfully employed to (*i*) identify a monoclonal antibody (MAb) reactive peptide that, sharing six (of 13) residues with the native antigen, could be used to induce antigen-reactive antibodies (Motti et al. 1994) and (*ii*) identify a MAb-reactive peptide that, sharing only three residues (over a 5-residue span) with the herpes simplex virus type 1 (HSV-1) native antigen, could when used as an immunogen protect mice against subsequent HSV-1 challenge (Schellekens et al. 1994). Conversely, the importance of a large and diverse library is suggested by the finding that, whereas antibody phage repertoires of $\sim 10^8$ clones have characteristically yielded antibody fragments of only moderate affinity ($\sim \mu M$), a 6.5×10^{10} clone repertoire provided antibodies to a range of antigens and haptens with affinities <10 nM (Griffiths et al. 1994).

That some biological bias exists in RPLs seems clear, as indicated by a study by Peters et al. (1994) where it was found that 5% of clones in a hexapeptide RPL produced 10- to 10^5-fold fewer virions than a parental (fd-tet) phage. What remains largely unclear (but see Peters et al. 1994) is the nature and origin of the bias.

The issue of position-specific bias against certain residues in the early mature region of pIII or pVIII fusion products has received little experimental attention. Although, in the single study of which we are aware, a careful analysis of 52 randomly selected decapeptide library clones found no evidence of position-specific bias (DeGraaf et al. 1993), it should be noted that random peptides in the library analysed were insulated from the pIII N-terminus by a relatively lengthy 7-residue linker sequence initiated by Ala–Asp (favoured at the N-terminus, as discussed earlier). Accordingly, the findings may not be broadly representative of other RPLs.

Crude measures of bias, derived from the overall amino acid content of a small number of randomly selected clones, suggest that small degrees of bias exist in some RPLs. Possibly, these reflect greater sequence- or position-specific effects or as suggested by one study (Bonnycastle et al. 1996), bias in oligonucleotide synthesis. Thus, various studies have reported or χ^2 analyses of their published data suggest, that (*i*) ~1.8-fold more Gly and Lys, and about half the expected Pro were represented in one RPL (Cwirla et al. 1990); (*ii*) more Gly and less Pro than expected were represented in certain other RPLs (Jellis et al. 1993; Burritt et al. 1996); and (*iii*) 2.1-fold more Gly (Dybwad et al. 1995*a*) and less than one-third the expected Cys (Dybwad et al. 1995*b*) were represented in other RPLs.

One study in which bias favouring Gly and disfavouring Cys were noted (Blond-Elguindi et al. 1993) also found that, in a manner consistent with their expected influence on membrane insertion and signal peptide cleavage, Asp and Glu were slightly more abundant towards the N-terminus, the reverse being true for Lys. Bias against Cys may be attributable to an idea put forth to explain an observed apparent selection against odd numbers of Cys residues (Kay et al. 1993); here it was reasoned that an unpaired Cys could form a bond with one of the eight native pIII Cys residues, thus affecting phage infectivity.

Representative results: affinity-selection with antibodies

A straightforward example of a successful RPL application is provided in a study (Conley et al. 1994) in which affinity-selection from a 15-residue RPL of limited diversity with an anti-human immunodeficiency virus-1 MAb yielded 20 MAb-reactive clones that could be grouped into the four consensus sequences XXDKW, $XLD^R/_KW$, $EXD^R/_KW$, and $ELD^R/_KW$ and thus be used to readily identify ELDKW as the native epitope. Not all RPL studies yield such apparent success. For example, affinity-selection from a hexapeptide RPL with MAbs against α-keratin yielded substantial enrichment for target clones for only one of seven MAbs (Böttger and Lane 1994). Although later studies with five MAbs (Böttger et al. 1995) had greater success with a 20-residue RPL than with a 12-residue RPL, the small number of 20-residue sequences recovered and their limited homology with the native protein were not enough to identify an epitope. Rather than a position-specific consensus sequence, a compositional theme emerged from a study (Sioud et al. 1994) employing human autoantibodies against tumor necrosis factor alpha. Thus, 45 of 63 affinity-selected clones analysed displayed the Ser- and Pro-rich sequences ASSLLASSP, NSSPYLNTK, or PQSPGSSFP and the remaining 17 clones also contained Ser or Pro.

Some RPL biopannings may yield misleading results. In discussing reasons for this, Smith and Scott (1993) have noted two consensus sequences, PWXWL and GDWVFI, that can arise from biopanning with both antibodies and other ligates and which may bind components of the biopanning system (e.g., streptavidin; Roberts et al. 1993). Indeed, phage display has been used to identify peptides that bind a number of such components, including streptavidin (Devlin et al. 1990; Kay et al. 1993), biotin (Saggio and Laufer 1993), mouse IgG Fc region (Kay et al. 1993), and plastic (Tyr- and Trp-rich sequences; see Adey et al. 1995). One study (Jellis et al. 1993) reported enrichment for both MAb-reactive target clones and clones bearing a streptavidin-binding motif (HPQN; Devlin et al. 1990), while another found that the common blocking agent bovine serum albumin could enrich for trypsin-displaying phage (Corey et al. 1993).

Affinity-selection with non-antibody molecules

As with antibodies, affinity-selection from RPLs with non-antibody molecules may yield peptides whose sequences make immediate and intuitive sense or which are quite unexpected. Thus, two groups (Cheadle et al. 1994; Sparks et al. 1994), who independently biopanned RPLs with fusion proteins containing the Src SH3 domain, identified similar proline-containing sequences related to a known SH3 domain binding motif. Similarly apparent success, in this case a collection of sequences containing the RGD triplet, resulted from affinity-selection from 6- and 15-residue RPLS with the integrin $\alpha_v\beta_3$ (Healy et al. 1995). In contrast, affinity-selection from a hexapeptide RPL with S-protein (a fragment of bovine pancreatic ribonuclease) identified a sequence motif, $^F/_YNF^E/_V{}^I/_V{}^L/_V$,

that bore little resemblance to S-protein's natural ligand (Smith et al. 1993). An even less sequence-specific and accordingly fitting motif was found by affinity-selection from 8- and 10-residue RPLs with the molecular chaperone BiP. The motif, $\Theta^{W}/_{X}\Theta X\Theta X\Theta$ (Θ = a large hydrophobic residue), fits well with the role of BiP in recognizing partially folded polypeptides (Blond-Elguindi et al. 1993). In this latter study, the use of ATP to elute target clones by stimulating the ATPase activity of BiP is noteworthy.

Affinity-selection from a 38-residue library with streptavidin (Kay et al. 1993) illustrates several aspects of the employment of RPLs. Of the two classes of sequences recovered, one provided a consensus sequence ($HP^{Q}/_{M}X$, where X = a nonpolar residue) and the other provided no clear motif. Intriguingly, clones of the nonmotif class bound to streptavidin with ~fivefold greater affinity than selected $HP^{Q}/_{M}X$ clones. An advantage of disulfide-constrained display was suggested by the finding that the affinity-selected sequence *CHPQAC* (* denotes potential disulfide bond partners) bound 100-fold less efficiently after treatment with a reducing agent.

Substrate phage

Substrate phage is an application of RPLs pioneered (Matthews and Wells 1993) to identify peptide substrates of proteases and which relies on the resistance of Ff phage to most proteases (Rasched and Oberer 1986). In a study (Matthews et al. 1994) that illustrates their use, a substrate phage library was constructed by inserting a randomized pentapeptide substrate flanked by di-alanine residues between a binding domain (a variant of human growth hormone; hGH) and pIII. After binding the phage to hGH receptor molecules, the library was treated with furin to release clones bearing a furin substrate. Six rounds of enrichment led to a consensus motif that was used as a seed for a second series of randomized libraries. Furin selection from these libraries yielded the motif $^{L}/_{P}RRF^{K}/_{R}RP$.

Success and failure

A theme common to both early (Cwirla et al. 1990; Devlin et al. 1990; Scott and Smith 1990) and more recent studies (e.g., Conley et al. 1994; Jellis et al. 1993; Kay et al. 1993) is that affinity-selection from RPLs can yield related sequences (e.g., as reviewed in Cortese et al. 1994, 1995) that as a collection provide insight (e.g., identification of residues that may be important to ligand–ligate interaction) not obtainable by methods (e.g., peptide scanning; Geysen et al. 1987) that lead to a single sequence. Yet a number of studies (e.g., Schellekens et al. 1994; Böttger and Lane 1994; Böttger et al. 1995; Yao et al. 1995; Wilson 1997; see also Lane and Stephen 1993) have not yielded the desired set of related sequences.

A number of technical and other reasons for apparent failures have been discussed (e.g., Smith and Scott 1993; Böttger and Lane 1994; Clackson and Wells 1994; Scott and Craig 1994; Schellekens et al. 1994; Bonnycastle et al. 1996). In some cases the solution may be straightforward. For example, epitopes recognized by some MAbs, such as the linear but discontinuous $KKX_{1-4}STSX_{0-4}HXK$ variants of a p53 epitope (Stephen et al. 1995), may be longer than those provided by the 6- to 9-residue libraries often employed, particularly in early studies. Thus, it can be hoped that a library of peptides of greater length will yield the desired target clones. Such libraries are now common. Other, more complex issues relate to the inherent diversity of the nature of ligand–ligate interactions. Accordingly, libraries displaying peptides in different structural contexts have been developed; some of these are discussed later.

Given a diversity of options and considering the reported failures, a key question for a researcher considering phage display for an application such as epitope identification is "what ensures success?" and an important task has thus been to extract the rules that govern this. For RPL applications involving antibodies, an important paper relevant to this task is that describing an extensive study (Bonnycastle et al. 1996) designed to examine the interplay among the natures of (*i*) the immunogen (peptide, protein, or carbohydrate), (*ii*) the epitope (linear or discontinuous), (*iii*) the antibody (monoclonal or polyclonal), and (*iv*) the phage-displayed peptide. Affinity-selection from 11 RPLs (displaying peptides of varying length in different structural contexts) with 17 antibodies led to the findings that (*i*) for a given antibody, not all libraries yielded a consensus sequence, (*ii*) in some cases, different libraries provided different consensus sequences or peptides with no apparent consensus, (*iii*) for most MAbs, the tightest-binding peptides were identified from a few libraries, and (*iv*) the libraries yielding these tight-binding peptides varied among MAbs (Bonnycastle et al. 1996). Thus, the diversity of factors involved in molecular interactions is reflected, in practical terms, in an inherent unpredictability.

Yet importantly, it was also found that, given an adequate set of libraries, reactive peptides could always be identified for antibodies elicited against peptides, linear epitopes of proteins, and carbohydrates but not always for antibodies against discontinuous epitopes of proteins. Thus, except for these latter epitopes, success can be assured, but it may require employment of multiple libraries. Although this is not so straightforward as may have been hoped for from the apparent simplicity of early phage display papers, perserverance may be sufficiently rewarding. For example, in a preface to two papers (Livnah et al. 1996; Wrighton et al. 1996) describing a peptide identified from phage libraries, Wells (1996) commented that the findings were "enough to reinstate one's belief in Santa Claus," for the authors had found a peptide able to form dimers and subsequently dimerize and activate the erythropoietin receptor. Described by Wells (1996) as "try everything you can think of," the successful strategy considered the relationships among such issues as methodology, affinity, and avidity and employed a number of pVIII and pIII libraries, as well as a variety of elution methods.

A final issue concerns our definition of success, which (in this review) has thus far required that phage libraries yield (*i*) sequences that correspond in some way with a native ligand or ligate (e.g., an epitope) or (*ii*) one or more collections of sequences for which consensus sequence(s) can be readily defined, but where there is no obvious homology to the native ligand or ligate (e.g., one or more mimotopes). This requirement derives from the idea that interactions are determined by molecular fit between ligand and ligate, such as by complementarity of shape and charge and hydrophilicity. Importantly, however, it has been found that interactions can also be mediated by a relatively large number of ordered water molecules at the ligand–ligate interface (e.g., Bhat et al. 1994). In such cases, where a molecular layer bridging ligand and ligate adds

a new level of complexity, it is conceivable that phage libraries will deliver a greater assortment of sequences, sufficiently diverse that success (now redefined in terms of finding reactive versus related sequences) may not be recognized as such.

Phage-displayed proteins and alternative methods of display

Phage display can be classified (Smith 1993) as Types 3, 3+3, 33, 8, 8+8, and 88, depending on the use of pIII or pVIII as the carrier molecule, the number of fusion products, and the vectors employed.

In the Type 3 systems of the first RPLs, a gIII cloning site in the phage genome enables display of a foreign peptide on each copy of pIII (Figs. 1C and 1D). In Type 3+3 display, recombinant gIII is encoded on a phagemid (a plasmid containing the Ff origins of replication and packaging signal). Here, superinfection of phagemid-harboring cells by a helper phage (an Ff phage with a defective packaging signal and interference-resistance mutation(s)) yields viral particles containing phagemid ss DNA and comprised of both wild-type and recombinant pIII. In Type 33 display, a single phage genome encodes both wild-type and recombinant gIII, the latter often being inserted into the multiple cloning site of an M13 cloning vector.

The relative merits of the pIII systems can be illustrated by three issues. The first, an issue in methodology, concerns intrinsic affinity versus functional avidity. Here it is reasoned (discussed in Wells and Lowman 1992) that target peptides affinity-selected from Type 3+3 PDLs will possess greater intrinsic affinity for their cognate molecule than those selected from Type 3 libraries where high functional avidity may derive from multiple low-affinity interactions. A second issue concerns the potential interference of fusions to pIII with its F-pilus adsorption function and consequent effects on phage infectivity. Here, interference can be minimized by Types 33 and 3+3 display, where wild-type pIII is incorporated into the viral particle. A third issue also concerns biology. Here it is hoped that reducing the level of expression of an intrinsically toxic or difficult-to-export pIII fusion protein will facilitate its display (e.g., Smith 1993; Cortese et al. 1994). Thus, larger polypeptides and proteins are often displayed with Types 33 and 3+3 vectors and for similar reasons low copy number vectors (fd-tet derivatives) may be beneficial in Type 3 display (Smith and Scott 1993). Although these approaches have intuitive merit, their general effectiveness has not been systematically addressed. Thus, success may vary from protein to protein and, in practical terms, will be readily determinable in applications involving display of a single protein or small number of variants but more difficult to assess for a genomic or other diverse library.

Types 8, 8+8, and 88 vectors are the pVIII counterparts to the Types 3, 3+3, and 33 vectors. Although Type 8 display is uncommon because of the difficulties, noted earlier, in displaying peptides longer than five or six residues, the recent generation of organic landscapes on phage using Type 8 display of octapeptides (Petrenko et al. 1996) points to their utility. Proteins as large as antibody Fab fragments have been displayed with Types 8+8 (Kang et al. 1991) and 88 (Huse et al. 1992) vectors. Importantly, like Type 3+3 vectors, Types 8+8 and 88 vectors can be employed for display in which ligand–ligate interactions are locally monovalent and may possess certain advantages over Type 3+3 vectors (Bonnycastle et al. 1996).

Type 3 display

Catalytically active *E. coli* alkaline phosphatase (AP) has been displayed in this way and, notably, catalytic activity required dimerization of AP-pIII on the phage surface (McCafferty et al. 1991). However, infectivity of the AP–pIII recombinants was <10% of that of the parent vector and proteolysis of the AP–pIII fusions was considerable. Only 5–10% of the fusions were intact in early preparations, 30–60% in later preparations. Similarly, in phage display of the 32-kDa B chain of ricin, the ratio of intact to proteolyzed B chain – pIII fusion products was 1:3 (Swimmer et al. 1992). Proteolysis was also suspected in Type 3 display of the 58-residue bovine pancreatic trypsin inhibitor (Roberts et al. 1992*b*). In contrast, no significant degradation was seen in display of fully active β-lactamase (Soumillion et al. 1994). That human interleukin 3 (hIL3) recombinants, displayed in a Type 3 vector (Gram et al. 1993), could be identified on the basis of their distinct small plaque phenotype points to an interplay between hIL3–pIII fusion protein and phage–host biology.

Type 3+3 display

Among early phage display papers was that describing phagemid display of the 191-residue disulfide-containing hGH, accomplished by fusion of hGH to the pIII C-terminal domain ($pIII_C$) required for virion morphogenesis (Bass et al. 1990). Importantly, hGH–$pIII_C$ could bind to hGH receptor and was recognized by MAbs whose epitopes are sensitive to hGH conformation and immunoblots showed little evidence of hGH–$pIII_C$ degradation. Although phagemid titers were expectedly sensitive to the fraction of recombinant pIII molecules incorporated, hGH–$pIII_C$ expression could be limited so that few phagemid particles displayed more than a single hGH–$pIII_C$ fusion protein. Other examples of Type 3+3 $pIII_C$ fusions include (*i*) the α subunit of the high-affinity receptor for IgE (FcεRI) in a form recognized by IgE (Scarselli et al. 1993) and (*ii*) the 23-kDa human ciliary neurotrophic factor in a form that bound to its natural receptor (Saggio et al. 1995). Degradation of the $pIII_C$ fusion proteins was not examined in these latter papers.

Other methods of Type 3+3 display involve fusions to complete mature pIII molecules. One such study involved display of the 33-kDa prostate specific antigen (PSA) in a form detectable by antibodies recognizing nonlinear epitopes (Eerola et al. 1994). Here, Western blotting showed degradation of many of the PSA–pIII fusion products. Staphylococcal protein A (SPA) has been displayed in this manner (Kushwaha et al. 1994) and it appears that most if not all of the phagemid-displayed SPA was of the expected size. Both Types 3 and 3+3 display of residues 1–176 of human CD4 produced $CD4_{1-176}$–pIII fusions in a form recognized by gp120 of the human immunodeficiency virus (Abrol et al. 1994). Published Western blots hint to proteolysis of some Type 3+3 $CD4_{1-176}$–pIII fusions. Infectivity of the Type 3 recombinants was reduced to 5% of that of the parent vector.

Types 33 and 88 display

In a study exploring both Types 33 and 88 display, sequences encoding a signal peptide and trypsin fused to $pIII_C$ or mature

pVIII cloned into the M13mp18 yielded virions comprised of both recombinant and wild-type coat protein (Corey et al. 1993). Interestingly, the endogenous *E. coli* protease inhibitor ecotin copurified with the tryspin-displaying virions and, importantly, trypsin-coat protein fusions possessed near wild-type trypsin activity. However, that <20% of virions displayed the fusions and mean infectivity (averaged for wild-type and recombinant virions) was <25% of that of the nonrecombinant vector suggest profound effects of the fusions on some aspect of host and phage biology.

The construction and employment of a Type 88 vector to display bovine pancreatic trypsin inhibitor (BPTI) illustrates several aspects of vector design and host biology. In a manner similar to that employed for trypsin display, sequences encoding BPTI–pVIII fusions were cloned into M13mp18 (Markland et al. 1991). Homologous recombination among recombinant and wild-type gVIII was avoided by the use of alternative codons to construct a synthetic gVIII for the BPTI fusions. The first constructs, encoding a fusion of the pVIII signal peptide, BPTI, and mature pVIII, produced no recombinant virions. Rather, Western blots of host cell lysates showed only a single pVIII-related protein with a size characteristic of unprocessed fusion protein. Use of an alternative signal sequence led to partial processing and reasonable levels of BPTI–pVIII incorporation into virions (≤2% of total pVIII) were eventually achieved by use of a *prlA* (*secY*) host. Replacing the ATG start codon of wild-type gVIII with CTG increased the fraction of BPTI–pVIII incorporated into virions to ~3% but reduced virion yields 10-fold.

Type 8+8 display

An early innovation involving Type 8+8 display is provided in a study (Kang et al. 1991; see also Barbas et al. 1991) that employed a phagemid to coexpress (*i*) antibody heavy chains (V_H–C_{H1}) as fusions between a PelB signal peptide and pVIII and (*ii*) light chains (V_L–C_L) as fusions to a second PelB signal peptide. The expectation that V_H–C_{H1}–pVIII and V_L–C_L molecules would associate in the periplasm to form properly folded Fab fragments were validated by studies including electron microscopy of phagemids decorated with antigen-Fab complexes.

Directed evolution of a protein

Phage display is popular for in vitro selection of peptides and proteins with altered properties, as suggested by the subheading "phage fever" in a review of the field (Clackson and Wells 1994). An early study, termed "directed evolution of a protein" (Roberts et al. 1992*a*), illustrates the approach. Here, a 1000-member PDL constructed by limited randomization of five residue positions of BPTI was biopanned with immobilized human neutrophil elastase (HNE) to yield a variant with $>10^6$-fold greater affinity for HNE than the parental BPTI sequence. Other studies, often with multiple libraries of greater diversity, have similarly yielded target clones with altered target specificity. These include not only other protease inhibitors (Dennis and Lazarus 1994*a*, 1994*b*; Wang et al. 1995*a*) but also an enzyme with altered specificity for active-site ligands (Widersten and Mannervik 1995) and zinc fingers with modified specificity in one or more fingers (Choo and Klug 1995).

Gene fragment libraries

Although the first PDL (Smith 1985) was a gene fragment library, studies employing gene fragment and genomic PDLs appear to be mainly limited to the following examples.

Multivalent pIII-display gene fragment libraries screened with antibodies

In an early application of gene fragment libraries (Bleul et al. 1991), a Type 3 library constructed with 200-bp DNase I generated fragments of subgenomic DNA of the human papilloma virus type 18 (HPV-18) was screened using plaque lifts (rather than biopanned) to identify two unique antibody-reactive peptides for each of two α-HPV-18 sera. Similarly, a relatively small (200 transformants) Type 3 library constructed with 100- to 200-bp fragments of the gene encoding the bluetongue virus capsid protein VP5 has been employed to identify, by affinity-selection, two overlapping (44- and 50-residue) peptides reactive with α-VP5 antibody (Wang et al. 1995*b*).

A more extensive study involved Type 3 libraries constructed with 50- to 200-bp gene fragments encoding peptides for (*i*) *Drosophila* RNA polymerase II, (*ii*) human 53 protein, and (*iii*) human cytokeratin 19 protein (Petersen et al. 1995). Epitopes recognized by MAbs raised against these proteins were identified after one or two rounds of affinity-selection. Interestingly, of the three cytokeratin 19 libraries constructed, only one yielded target clones; here, Cys-containing linkers had been used to present peptides in a disulfide-constrained loop.

Identification of ligand-binding domains with a phagemid system

Type 3+3 display has also been employed for gene fragment libraries. In a model system exploring the feasibility of prokaryotic genomic libraries, a phagemid-based library constructed with 100- to 700-bp sonication products of *Staphylococcus aureus* genomic DNA was biopanned with human IgG and fibronectin and subsequently screened by colony blotting and hybridization with oligonucleotide probes to identify known binding regions of staphylococcal protein A and fibronectin-binding proteins (Jacobsson and Frykberg 1995). Curiously, +1 or −1 frameshifts were found in the cloned inserts of all five fibronectin-binding clones and in related work, 47 of 50 clones derived from other libraries possessed similar frameshifts. Frameshifts were also noted in studies employing Type 8+8 display (Jacobsson and Frykberg 1996).

cDNA libraries by means of Jun and Fos leucine zippers

In a manner similar to an approach described earlier for Fab display (Barbas et al. 1991; Kang et al. 1991), an innovative phagemid system for display of cDNA fragments exploits the interaction of Jun and Fos leucine zippers (Crameri and Suter 1993; Crameri et al. 1994). Here, expression of *pelB–jun–gIII*$_C$ and *pelB–fos*–cDNA fusions and the resulting intraperiplasmic high-affinity Fos–Jun interactions (and subsequent covalent linkage mediated by engineered disulfide bonds) allows display of cDNA-derived peptides on a phagemid surface. Importantly, display involves C-terminal fusions to Fos rather than insertions near the pIII N-terminus. Thus, (*i*) cDNA or other gene fragments containing translation stop sites or other noncoding sequences can be expressed and (*ii*) the N-terminal

mature region of the fusion proteins, believed important in membrane translocation of proteins, is constant among library members.

Affinity-selection from gene fragment libraries

The heterologous nature (in size and sequence) of displayed peptides sets gene fragment libraries apart from other PDLs, which characteristically display (*i*) peptides of enormous diversity but of small size, as in RPLs or (*ii*) large peptides or proteins in which a small number of residues are varied within an otherwise uniform scaffold, as in antibody display. Since clonal competition may thus prove to be relatively greater in gene fragment PDLs, conventional methods of affinity-selection, which include library amplification between multiple rounds of enrichment, may be inappropriate.

In this context, it may be relevant that several applications of gene fragment libraries reviewed above (*i*) avoided affinity-enrichment and instead used direct screening by plaque lifts (Bleul et al. 1991) or (*ii*) employed only one or two rounds (Petersen et al. 1995) of affinity-enrichment followed by screening. In studies that employed PDLs constructed with DNase I generated fragments of the gene encoding *B. pertussis* filamentous hemagglutinin, we similarly employed a single affinity-enrichment followed by screening by plaque lifts (Wilson 1997; Wilson et al. 1997). As we found in follow-up studies, clonal competition during library amplification does favour growth of nonrecombinants or other clones not recognized by antibody but not to the extent of significantly negating the gains from enrichment (Wilson 1997). Thus, our caution may have been unnecessary. However, in studies of other gene fragment libraries, virion yields were found to vary 100-fold among clones, in some cases 10^7-fold (Wilson 1997). With the latter libaries, clonal competition would expectedly be an issue.

Innovations in peptide presentation

Several methods have been developed to display peptides in a constrained, often defined, conformation. The simplest of these employs a disulfide-bonded loop. Other methods involve peptide display on a relatively larger molecular scaffold.

Disulfide-constrained peptides

Because virion morphogenesis involves insertion of pIII and pVIII into the inner membrane with their N-termini within the periplasm, paired cysteines flanking randomized sequences (e.g., *CX_6C*, where * denotes disulfide bond partners) can be used to present peptides in a disulfide-bonded loop (Luzzago et al. 1993; McLafferty et al. 1993; Zhong et al. 1994). Cys-constrained RPLs have been employed with remarkable success. The earlier-described identification of a 20-residue disulfide-constrained peptide capable of activating the erythropoietin receptor is one example. The isolation of brain- and kidney-targeting phage after intravenous injection of Cys-constrained RPLs into mice (Pasqualini and Ruoslahti 1996) is another novel illustration.

The relative merits of disulfide constraint can be illustrated by more conventional studies. In one application, a disulfide bond was required for high-affinity binding of MAb-reactive peptides identified by affinity-selection from a X*CX_4C*X RPL (McLafferty et al. 1993). In another, a disulfide bridge was required for binding of affinity-selected clones to a conformation-dependent MAb but not for binding to nonconformation-dependent MAbs (Zhong et al. 1994). The latter study also found that Cys-constrained peptides possessed greater antigenic fitness (the ability of a peptide to elicit an immune response against the native antigen) than unconstrained peptides.

In contrast, work that involved affinity-selection from both unconstrained (X_8) and constrained (*CX_6C*) libraries (McConnell et al. 1994) found that while the X_8 library yielded peptides with residues (VYIH) corresponding to those of the native antigen, the *CX_6C* library yielded only a mimotope sequence (DWWH). Moreover, follow-up studies showed that YVIH, when constrained within a disulfide-bonded loop, possessed considerably reduced affinity for the cognate MAb. Finally, a further study (McConnell and Hoess 1995) identified the MAb-reactive consensus sequence LEPW from both constrained and unconstrained RPLs. Notably, Bonnycastle et al. (1996) have found that the preference of an antibody for a specific type of disulfide constraint, or no constraint at all, is essentially unpredictable.

In reviewing constrained display, Ladner (1995) has suggested that increasing the disulfide-imposed constraint imposed on a peptide segment (e.g., RGD in XXXRGDXXX, *CXXRGDXXC*, and *CX*CRGDC*XC*) decreases the likelihood that it will bind to any particular target, but if a target is found, the peptide will bind more tightly and specifically.

Presentation on carrier molecules

Tendamistat, a 74 amino acid molecule comprised of two three-strand β-sheets, has been employed as a scaffold in which two adjacent strand-connecting loops (13 and 6 residues) can be randomized (McConnell and Hoess 1995) in Type 33 display. An expected advantage of constrained display involving two (versus one) randomized loops is the better approximation of surface structures involved in intermolecular recognition. The 61-residue minibody (Pessi et al. 1993) is similarly comprised of two three-strand β-sheets and retains the regions corresponding to two adjacent hypervariable loops (H1 and H2) of the antibody V_H domain from which it was engineered. Its utility was demonstrated (Martin et al. 1994) by the identification from a Type 3 library of a variant whose H2 region shared homology with a portion of the IL-6Rα receptor and which inhibited the biological activity of human interleukin-6. A more potent variant was identified by Type 3+3 minibody display in which the H1 loop of the previously identified H2 variant was randomized (Martin et al. 1996).

Phage display of randomized α-helices has been explored in a study (Bianchi et al. 1995) in which five helix positions (that are most exposed in the helix and cluster on one side) of a 26-residue zinc finger were randomized. Notably, the coupling of zinc coordination and folding in zinc fingers allows zinc-dependent binding to be used as a control against structurally undefined sequences. Phage display involving randomizing residues of a surface comprised of two α-helices of a monovalent Fc-binding domain of staphylococcal protein A has also been explored (Nord et al. 1995, 1997).

Innovations in identification of target clones

Several methods to identify target clones exploit the periplasmic association of separately translocated molecules (reviewed earlier) to form a complex that becomes incorporated into a virion. The innovation lies in the physical division of pIII into its two functional domains, the N-terminal ($pIII_N$) adsorption domain, and the C-terminal ($pIII_C$) morphogenetic domain.

In the direct interaction rescue system (Gramatikoff et al. 1994, 1995), an invariant bait molecule such as c-Jun is cloned as an N-terminal fusion to $pIII_C$ and variant fragments such as those from a human cDNA library are cloned as C-terminal fusions to $pIII_N$. In cells harbouring a cDNA fragment encoding a peptide that interacts with the Jun bait (Fos was the target in the model system described), periplasmic association of Jun–$pIII_C$ and $pIII_N$–cDNA fusion products yields infectious virions constructed with the heterodimeric complexes; in other cells, noninfectious virions containing only Jun–$pIII_C$ are produced. The similar selectively-infective phage system (Krebber et al. 1995) employs single-chain antibodies (scFv)–$pIII_C$ and $pIII_N$–antigen fusions. Infectivity is restored by cognate interactions between $pIII_N$–antigen and scFv–$pIII_C$. Notably, antigen or antibody sequences can be randomized or otherwise varied. A similar system has been developed (Duenas and Borrebaeck 1994) to mimic clonal selection by the immune system.

Concluding remarks

Ff phage display has been notably successful, even to the extent of enabling research into questions not readily answerable by other means. Yet not all ventures in this evolving field have been (entirely) successful. Among the reasons for this, as we have argued, are that phage and host cell biology may impose greater limitations than we initially may have hoped for.

In part because of such limitations, promising systems employing lytic phage such as λ and T4 have been developed (Maruyama et al. 1994; Dunn 1995; Efimov et al. 1995; Sternberg and Hoess 1995; Mikawa et al. 1996; see also Cortese et al. 1996). The seemingly clear advantage to lytic phage display is that incorporation of recombinant proteins into virion coats or tail proteins does not require the host's membrane translocation apparatus or other aspects of membrane-mediated assembly and export. Nevertheless, lytic phage display will likely prove to have its own characteristic shortcomings and Ff and lytic phage display can thus be expected to provide complementary and increasingly useful tools for research.

At present we have only a largely general understanding of the ways in which Ff phage and host biology must (in principle) and do (in experience) impose limitations on successful phage display. Hopefully, future studies will provide greater insight into the ways in which a displayed peptide or protein influences its ability to be successfully displayed for, as our understanding increases, so will the utility of phage display.

Acknowledgments

Research in our laboratory that has been cited in this review was supported by a Natural Sciences and Engineering Research Council of Canada 1967 Science and Engineering Scholarship awarded to D.R.W. and an operating grant to B.B.F. from the Canadian Bacterial Diseases Network Centre of Excellence.

References

Abbot, P.J. 1985. Stimulation of recombination between homologous sequences on carcinogen-treated plasmid DNA and chromosomal DNA by induction of the SOS response in *Escherichia coli* K12. Mol. Gen. Genet. **201**: 129–132.

Abrol, S., Sampath, A., Arora, K., and Chaudhary, V.K. 1994. Construction and characterization of M13 bacteriophages displaying gp120 binding domains of human CD4. Indian J. Biochem. Biophys. **31**: 302–309.

Adey, N.B., Mataragnon, A.H., Rider, J.E., Carter, J.M., and Kay, B.K. 1995. Characterization of phage that bind plastic from phage-displayed random peptide libraries. Gene, **156**: 27–31.

Andersson, H., and von Heijne, G. 1991. A 30-residue-long "export initiation domain" adjacent to the signal sequence is critical for protein translocation across the inner membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **88**: 9751–9754.

Armstrong, J., Perham, R.N., and Walker, J.E., 1981. Domain structure of bacteriophage fd adsorption protein. FEBS Lett. **135**: 167–172.

Barbas, C.F.I. 1993. Recent advances in phage display. Curr. Opin. Biotechnol. **4**: 526–530.

Barbas, C.F.I., Kang, A.S., Lerner, R.A., and Benkovic, S.J. 1991. Assembly of combinatorial libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. U.S.A. **88**: 7978–7982.

Bass, S., Greene, R., and Wells, J.A. 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. Proteins, **8**: 309–314.

Berg, O.G., and Silva, P.J.N. 1997. Codon bias in *Escherichia coli*: the influence of codon context on mutation and selection. Nucleic Acids Res. **25**: 1397–1404.

Bhat, T.N., Bentley, G.A., Boulot, G., Greene, M.I., Tello, D., Dall'Acqua, W., Souchon, H., Schwarz, F.P., Mariuzza, R.A., and Poljak, R.J. 1994. Bound water molecules and conformational stabilization help mediate an antigen-antibody association. Proc. Natl. Acad. Sci. U.S.A. **91**: 1089–1093.

Bianchi, E., Folgori, A., Wallace, A., Nicotra, M., Acali, S., Phalipon, A., Barbato, G., Bazzo, R., Cortese, R., Felici, F., and Pessi, A. 1995. A conformationally homogenous combinatorial peptide library. J. Mol. Biol. **247**: 154–160.

Bleul, C., Muller, M., Frank, R., Gausepohl, H., Koldovsky, U., Mgaya, H.N., Luande, J., Pawlita, M., ter Meulen, J., Viscidi, R., and Gissmann, L. 1991. Human papillomavirus type 18 E6 and E7 antibodies in human sera: increased anti-E7 prevalence in cervical cancer patients. J. Clin. Microbiol. **29**: 1579–1588.

Blond-Elguindi, S., Cwirla, S.E., Dower, W.J., Lipshutz, R.J., Sprang, S.R., Sambrook, J.F., and Gething, M.-J.H. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell, **75**: 717–728.

Boeke, J.D., Russel, M., and Model, P. 1980. Processing of filamentous phage pre-coat protein. Effect of sequence variations near the signal peptidase cleavage site. J. Mol. Biol. **144**: 103–116.

Boeke, J.D., Model, P., and Zinder, N.D. 1982. Effects of bacteriophage f1 gene III protein on the host cell membrane. Mol. Gen. Genet. **186**: 185–192.

Bonnycastle, L.L.C., Mehroke, J.S., Rashed, M., Gong, X., and Scott, J.K. 1996. Probing the basis of antibody reactivity with a panel of constrained peptide libraries displayed by filamentous phage. J. Mol. Biol. **258**: 747–762.

Böttger, V., and Lane, E.B. 1994. A monoclonal antibody epitope on keratin 8 identified using a phage peptide library. J. Mol. Biol. **235**: 61–67.

Böttger, V., Stasiak, P.C., Harrison, D.L., Mellerick, D.M., and Lane, E.B. 1995. Epitope mapping of monoclonal antibodies to keratin

19 using keratin fragments, synthetic peptides and phage peptide libraries. Eur. J. Biochem. **231**: 475–485.

Boyd, D., and Beckwith, J. 1990. The role of charged amino acids in the localization of secreted and membrane proteins. Cell, **62**: 1031–1033.

Bradbury, A., and Cattaneo, A. 1995. The use of phage display in neurobiology. Trends Neurosci. **18**: 243–249.

Burritt, J.B., Bond, C.W., Doss, K.W., and Jesaitis, A.J. 1996. Filamentous phage display of oligopeptide libraries. Anal. Biochem. **238**: 1–13.

Cao, G., Kuhn, A., and Dalbey, R.E. 1995. The translocation of negatively charged residues across the membrane is driven by the electrochemical potential: evidence for an electrophoresis-like membrane transfer mechanism. EMBO J. **14**: 866–875.

Cheadle, C., Ivashchenko, Y., South, V., Searfoss, G.H., French, S., Howk, R., Ricca, G.A., and Jaye, M. 1994. Identification of a Src SH3 domain binding motif by screening a random phage display library. J. Biol. Chem. **269**: 24 034 – 24 039.

Choo, Y., and Klug, A. 1995. Design DNA-binding proteins on the surface of filamentous phage. Curr. Opin. Biotechnol. **6**: 431–436.

Clackson, T., and Wells, J.A. 1994. In vitro selection from protein and peptide libraries. Trends Biotechnol. **12**: 173–184.

Conley, A.J., Kessler, J.A.I., Boots, L.J., Tung, J.-S., Arnold, B.A., Keller, P.M., Shaw, A.R., and Emini, E.A. 1994. Neutralization of divergent human immunodeficiency virus type 1 variants and primary isolates by IAM-41–2F5, an anti-gp41 human monoclonal antibody. Proc. Natl. Acad. Sci. U.S.A. **91**: 3348–3352.

Corey, D.R., Shiau, A.K., Yang, Q., Janowski, B.A., and Craik, C.S. 1993. Trypsin display on the surface of bacteriophage. Gene, **128**: 129–134.

Cortese, R., Felici, F., Galfre, G., Luzzago, A., Monaci, P., and Nicosia, A. 1994. Epitope discovery using peptide libraries displayed on phage. Trends Biotechnol. **12**: 262–267.

Cortese, R., Monaci, P., Nicosia, A., Luzzago, A., Felici, F., Galfre, G., Pessi, A., Tramontano, A., and Sollazzo, M. 1995. Identification of biologically active peptides using random libraries displayed on phage. Curr. Opin. Biotechnol. **6**: 73–80.

Cortese, R., Monaci, P., Luzzago, A., Santini, C., Bartoli, F., Cortese, I., Fortugno, P., Galfre, G., Nicosia, A., and Felici, F. 1996. Selection of biologically active peptides by phage display of random peptide libraries. Curr. Opin. Biotechnol. **7**: 616–621.

Crameri, R., and Suter, M. 1993. Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. Gene, **137**: 69–75.

Crameri, R., Jaussi, R., Menz, G., and Blaser, K. 1994. Display of expression products of cDNA libraries on phage surfaces. A versatile screening system for selective isolation of genes by specific gene-product/ligand interaction. Eur. J. Biochem. **226**: 53–58.

Crissman, J.W., and Smith, G.P. 1984. Gene-III protein of filamentous phages: evidence for a carboxyl-terminal domain with a role in morphogenesis. Virology, **132**: 445–455.

Cwirla, S.E., Peters, E.A., Barrett, R.W., and Dower, W.J. 1990. Peptides on phage: a vast library for identifying ligands. Proc. Natl. Acad. Sci. U.S.A. **87**: 6378–6382.

DeGraaf, M.E., Miceli, R.M., Mott, J.E., and Fischer, H.D. 1993. Biochemical diversity in a phage display library of random decapeptides. Gene, **128**: 13–17.

Dennis, M.S., and Lazarus, R.A. 1994*a*. Kunitz domain inhibitors of tissue factor-factor VIIa. I. Potent inhibitors selected from libraries by phage display. J. Biol. Chem. **269**: 22 129 – 22 136.

Dennis, M.S., and Lazarus, R.A. 1994*b*. Kunitz domain inhibitors of tissue factor-factor VIIa. II. Potent and specific inhibitors by competitive phage selection. J. Biol. Chem. **269**: 22 137 – 22 144.

Devlin, J.J., Panganiban, L.C., and Devlin, P.E. 1990. Random peptide libraries: a source of specific protein binding molecules. Science (Washington, D.C.), **249**: 404–406.

Dolzani, L., Lagatolla, C., and Monti-Bragadin, C. 1991. Prevalence of recombinational versus mutational events in damaged plasmid DNA containing regions of homology with the chromosome. Mutat. Res. **264**: 127–134.

Dong, H., Nilsson, L., and Kurland, C.G. 1996. Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates. J. Mol. Biol. **260**: 649–663.

Driessen, A.J.M. 1994. How proteins cross the bacterial cytoplasmic membrane. J. Membr. Biol. **142**: 145–159.

Duenas, M., and Borrebaeck, C.A. 1994. Clonal selection and amplification of phage displayed antibodies by linking antigen recognition and phage replication. Bio/Technology, **12**: 999–1002.

Dunn, I.S. 1995. Assembly of functional bacteriophage lambda virions incorporating C-terminal peptide or protein fusions with the major tail protein. J. Mol. Biol. **248**: 497–506.

Dunn, I.S. 1996. Phage display of proteins. Curr. Opin. Biotechnol. **7**: 547–553.

Dybwad, A., Bogen, B., Natvig, J.B., Forre, O., and Sioud, M. 1995*a*. Peptide phage libraries can be an efficient tool for identifying antibody ligands for polyclonal antisera. Clin. Exp. Immunol. **102**: 438–442.

Dybwad, A., Forre, O., Natvig, J.B., and Sioud, M. 1995*b*. Structural characterization of peptides that bind synovial fluid antibodies from RA patients: a novel strategy for identification of disease-related epitopes using a random peptide library. Clin. Immunol. Immunopathol. **75**: 45–50.

Eerola, R., Saviranta, P., Lilja, H., Pettersson, K., Lovgren, T., and Karp, M. 1994. Expression of prostate specific antigen on the surface of a filamentous phage. Biochem. Biophys. Res. Commun. **200**: 1346–1352.

Efimov, V.P., Nepluev, I.V., and Mesyanzhinov, V.V. 1995. Bacteriophage T4 as a surface display vector. Virus Genes, **10**: 173–177.

Endemann, H., and Model, P. 1995. Location of filamentous phage minor coat proteins in phage and in infected cells. J. Mol. Biol. **250**: 496–506.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., and Cesareni, G. 1991. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. J. Mol. Biol. **222**: 301–310.

Feng, J.N., Russel, M., and Model, P. 1997. A permeabilized cell system that assembles filamentous bacteriophage. Proc. Natl. Acad. Sci. U.S.A. **94**: 4068–4073.

Fulford, W., and Model, P. 1988*a*. Bacteriophage f1 DNA replication genes. II. The roles of gene V protein and gene II protein in complementary strand synthesis. J. Mol. Biol. **203**: 39–48.

Fulford, W., and Model, P. 1988*b*. Regulation of bacteriophage f1 DNA replication. I. New functions for genes II and X. J. Mol. Biol. **203**: 49–62.

Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G., and Schoofs, P.G. 1987. Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods, **102**: 259–274.

Gram, H., Stritmatter, U., Lorenz, M., Gluck, D., and Zenke, G. 1993. Phage display as a rapid gene expression system: production of bioactive cytokine-phage and generation of neutralizing monoclonal antibodies. J. Immunol. Methods, **161**: 169–176.

Gramatikoff, K., Georgiev, O., and Schaffner, W. 1994. Direct interaction rescue, a novel filamentous phage technique to study protein-protein interactions. Nucleic Acids Res. **22**: 5761–5762.

Gramatikoff, K., Schaffner, W., and Georgiev, O. 1995. The leucine zipper of c-Jun binds to ribosomal protein L18a: a role in Jun protein regulation. Biol. Chem. Hoppe-Seyler **376**: 321–325.

Greenwood, J., Willis, A.E., and Perham, R.N. 1991. Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from *Plasmodium falciparum* sporozoite protein as antigens. J. Mol. Biol. **220**: 821–827.

Griffiths, A.D., Williams, S.C., Hartley, O., Crosby, W.L., Konter-

mann, R.E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E., and Winter, G. 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. **13**: 3245–3260.

Grihalde, N.D., Chen, Y.-C.J., Golden, A., Gubbins, E., and Mandecki, W. 1995. Epitope mapping of anti-HIV and anti-HCV monoclonal antibodies and characterization of epitope mimics using a filamentous phage peptide library. Gene, **166**: 187–195.

Hart, S.L., Knight, A.M., Harbottle, R.P., Mistry, A., Hunger, H.-D., Cutler, D.F., Williamson, R., and Coutelle, C. 1994. Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. J. Biol. Chem. **269**: 12 468 – 12 474.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. Nature (London), **381**: 571–580.

Healy, J.M., Murayama, O., Maeda, T., Yoshino, K., Sekiguchi, K., and Kikuchi, M. 1995. Peptide ligands for integrin $\alpha_v\beta_3$ selected from random phage display libraries. Biochemistry, **34**: 3948–3955.

Hesterkamp, T., and Bukau, B. 1996. The *Escherichia coli* trigger factor. FEBS Lett. **389**: 32–34.

Higashitani, N., Higashitani, A., Roth, M., and Horiuchi, K. 1992. SOS induction in *Escherichia coli* by infection with mutant filamentous phage that are defective in initiation of complementary-strand DNA synthesis. J. Bacteriol. **174**: 1612–1618.

Higashitani, N., Higashitani, A., and Horiuchi, K. 1995. SOS induction in *Escherichia coli* by single-stranded DNA of mutant filamentous phage: monitoring by cleavage of LexA repressor. J. Bacteriol. **177**: 3610–3612.

Higashitani, A., Higashitani, N., and Horiuchi, K. 1997. Minus-strand origin of filamentous phage versus transcriptional promoters in recognition of RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **94**: 2909–2914.

Huse, W.D., Stinchcombe, T.J., Glaser, S.M., Starr, L., MacLean, M., Hellstrom, K.E., Hellstrom, I., and Yelton, D.E. 1992. Application of a filamentous phage pVIII fusion protein system suitable for efficient production, screening, and mutagenesis of F(ab) antibody fragments. J. Immunol. **149**: 3914–3920.

Hutchinson, F. 1996. Mutagenesis. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. *Edited by* F.C. Neidhardt. ASM Press, Washington, D.C. pp. 2218–2235.

Iannolo, G., Minenkova, O., Petruzzelli, R., and Cesareni, G. 1995. Modifying filamentous phage capsid: limits in the size of the major capsid protein. J. Mol. Biol. **248**: 835–844.

Izard, J.W., and Kendall, D.A. 1994. Signal peptides: exquisitely designed transport promoters. Mol. Microbiol. **13**: 765–773.

Jacobsson, K., and Frykberg, L. 1995. Cloning of ligand-binding domains of bacterial receptors by phage display. Biotechniques, **18**: 878–885.

Jacobsson, K., and Frykberg, L. 1996. Phage display shot-gun cloning of ligand-binding domains of prokaryotic receptors approaches 100% clones. Biotechniques, **20**: 1070–1081.

Jellis, C.L., Cradick, T.J., Rennert, P., Salinas, P., Boyd, J., Amirault, T., and Gray, G.S. 1993. Definining critical residues in the epitope for a HIV-neutralizing monoclonal antibody using phage display and peptide array technologies. Gene, **137**: 63–68.

Kaiser, C.A., Preuss, D., Grisafi, P., and Botstein, D. 1987. Many random sequences functionally replace the secretion signal sequence of yeast invertase. Science (Washington, D.C.), **235**: 312–317.

Kane, J.F. 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. Curr. Opin. Biotechnol. **6**: 494–500.

Kang, A.S., Barbas, C.F., Janda, K.D., Benkovic, S.J., and Lerner, R.A. 1991. Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. Proc. Natl. Acad. Sci. U.S.A. **88**: 4363–4366.

Kay, B.K., Adey, N.B., He, Y.-S., Manfredi, J.P., Mataragnon, A.H., and Fowlkes, D.M. 1993. An M13 phage library displaying random 38-amino-acid peptides as a source of novel sequences with affinity to selected targets. Gene, **128**: 59–65.

Kim, M.H., Hines, J.C., and Ray, D.S. 1981. Viable deletions in the M13 complementary strand origin. Proc. Natl. Acad. Sci. U.S.A. **78**: 6784–6788.

Krebber, C., Spada, S., Desplancq, D., and Pluckthun, A. 1995. Co-selection of cognate antibody-antigen pairs by selectively-infective phages. FEBS Lett. **377**: 227–231.

Kuhn, A. 1995. Major coat proteins of bacteriophage Pf3 and M13 as model systems for Sec-independent protein transport. FEMS Microbiol. Rev. **17**: 185–190.

Kushwaha, A., Chowdhury, P.S., Arora, K., Abrol, S., and Chaudhary, V.K. 1994. Construction and characterization of M13 bacteriophages displaying functional IgG-binding domains of Staphylococcal protein A. Gene, **151**: 45–51.

Ladner, R.C. 1995. Constrained peptides as binding entities. Trends Biotechnol. **13**: 426–430.

Lah, M., Goldstraw, A., White, J.F., Dolezal, O., Malby, R., and Hudson, P.J. 1994. Phage surface presentation and secretion of antibody fragments. Hum. Antibod. Hybrid. **5**: 48–56.

Lane, D.P., and Stephen, C.W. 1993. Epitope mapping using bacteriophage peptide libraries. Curr. Opin. Immunol. **5**: 268–271.

Li, P., Beckwith, J., and Inouye, H. 1988. Alteration of the amino terminus of the mature sequence of a periplasmic protein can severely affect protein export in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **85**: 7685–7689.

Livnah, O., Stura, E.A., Johnson, D.L., Middleton, S.A., Mulcahy, L.S., Wrighton, N.C., Dower, W.J., Jolliffe, L.K., and Wilson, I.A. 1996. Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 Å. Science (Washington, D.C.), **273**: 464–471.

Lloyd, R.G., and Low, K.B. 1996. Homologous recombination. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. *Edited by* F.C. Neidhardt. ASM Press, Washington, D.C. pp. 2236–2255.

Luzzago, A., Felici, F., Tramontano, A., Pessi, A., and Cortese, R. 1993. Mimicking of discontinuous epitopes by phage-displayed peptides. I. Epitope mapping of human H ferritin using a phage library of constrained peptides. Gene, **128**: 51–57.

MacIntyre, S., and Henning, U. 1990. The role of the mature part of secretory proteins in translocation across the plasma membrane and in regulation of their synthesis in *Escherichia coli*. Biochimie, **72**: 157–167.

Malik, P., Terry, T.D., Gowda, L.R., Langara, A., Petukhov, S.A., Symmons, M.F., Welsh, L.C., Marvin, D.A., and Perham, R.N. 1996. Role of capsid structure and membrane protein processing in determining the size and copy number of peptides displayed on the major coat protein of filamentous bacteriophage. J. Mol. Biol. **260**: 9–21.

Markland, W., Roberts, B.L., Saxena, M.J., Guterman, S.K., and Ladner, R.C. 1991. Design, construction and function of a multicopy display vector using fusions to the major coat protein of bacteriophage M13. Gene, **109**: 13–19.

Marks, J.D., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. 1992. Molecular evolution of proteins on filamentous phage: mimicking the strategy of the immune system. J. Biol. Chem. **267**: 16 007 – 16 010.

Martin, F., Toniatti, C., Salvati, A.L., Venturini, S., Ciliberto, G., Cortese, R., and Sollazzo, M. 1994. The affinity-selection of a minibody polypeptide inhibitor of human interleukin-6. EMBO J. **13**: 5303–5309.

Martin, F., Toniatti, C., Salvati, A.L., Ciliberto, G., Cortese, R., and Sollazzo, M. 1996. Coupling protein design and in vitro selection strategies: improving specificity and affinity of a designed β-protein IL-6 antagonist. J. Mol. Biol. **255**: 86–97.

Maruyama, I.N., Maruyama, H.I., and Brenner, S. 1994. Lambda foo: a lambda phage vector for the expression of foreign proteins. Proc. Natl. Acad. Sci. U.S.A. **91**: 8273–8277.

Marvin, D.A., Hale, R.D., Nave, C., and Helmer Citterich, M. 1994. Molecular models and structural comparisons of native and mutant class I filamentous bacteriophages Ff (fd, f1, M13), If1 and IKe. J. Mol. Biol. **235**: 260–286.

Matthews, D.J., and Wells, J.A. 1993. Substrate phage: selection of protease substrates by monovalent phage display. Science (Washington, D.C.), **260**: 1113–1117.

Matthews, D.J., Goodman, L.J., Gorman, C.M., and Wells, J.A. 1994. A survey of furin substrate specificity using substrate phage display. Protein Sci. **3**: 1197–1205.

Mayhew, M., and Hartl, F.-U. 1996. Molecular chaperone proteins. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. *Edited by* F.C. Neidhardt. ASM Press, Washington, D.C. pp. 922–937.

McCafferty, J., Griffiths, A.D., Winter, G., and Chiswell, D.J. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature (London), **348**: 552–554.

McCafferty, J., Jackson, R.H., and Chiswell, D.J. 1991. Phage-enzymes: expression and affinity chromatography of functional alkaline phosphatase on the surface of bacteriophage. Protein Eng. **4**: 955–961.

McConnell, S.J., and Hoess, R.H. 1995. Tendamistat as a scaffold for conformationally constrained phage peptide libraries. J. Mol. Biol. **250**: 460–470.

McConnell, S.J., Kendall, M.L., Reilly, T.M., and Hoess, R.H. 1994. Constrained peptide libraries as a tool for finding mimotopes. Gene, **141**: 115–118.

McLafferty, M.A., Kent, R.B., Ladner, R.C., and Markland, W. 1993. M13 bacteriophage displaying disulfide-constrained microproteins. Gene, **128**: 29–36.

Mikawa, Y.G., Maruyama, I.N., and Brenner, S. 1996. Surface display of proteins on bacteriophage lambda heads. J. Mol. Biol. **262**: 21–30.

Miller, C.G. 1996. Protein degradation and proteolytic modification. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. *Edited by* F.C. Neidhardt. ASM Press, Washington, D.C. pp. 938–954.

Miller, J.H., and Low, K.B. 1984. Specificity of mutagenesis resulting from the induction of the SOS system in the absence of mutagenic treatment. Cell, **37**: 675–682.

Model, P., and Russel, M. 1988. Filamentous bacteriophage. *In* The bacteriophages, *Edited by* R. Calendar. Plenum Press, New York. pp. 375–456.

Motti, C., Nuzzo, M., Meola, A., Galfre, G., Felici, F., Cortese, R., Nicosia, A., and Monaci, P. 1994. Recognition by human sera and immunogenicity of HBsAg mimotopes selected from an M13 phage display library. Gene, **146**: 191–198.

Mudgett, J.S. 1991. Ultraviolet light-induced plasmid-chromosome recombination in *Escherichia coli*: the role of *recB* and *recF*. Gene, **97**: 131–136.

Murphy C.K. and Beckwith J. 1996. Export of proteins to the cell envelope in *Escherichia coli*. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. *Edited by* F.C. Neidhardt. ASM Press, Washington, D.C. pp. 967–1004.

Nelson, F.N., Friedman, S.M., and Smith, G.P. 1981. Filamentous phage DNA cloning vectors: a noninfective mutant with a nonpolar deletion in gene III. Virology, **108**: 338–350.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**: 1–6.

Nord, K., Nilsson, J., Nilsson, B., Uhlen, M., and Nygren, P.-A. 1995. A combinatorial library of an α-helical bacterial receptor domain. Protein Eng. **8**: 601–608.

Nord, K., Gunneriusson, E., Ringdahl, J., Ståhl, S., Uhlén, M., and Nygren, P.-A. 1997. Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain. Nature Biotechnol. **15**: 772–777.

O'Neil, K.T., and Hoess, R.H. 1995. Phage display: protein engineering by directed evolution. Curr. Opin. Struct. Biol. **5**: 443–449.

Parmley, S.F., and Smith, G.P. 1988. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene. **73**: 305–318.

Pasqualini, R., and Ruoslahti, E. 1996. Organ targeting in vivo using phage display peptide libraries. Nature (London), **380**: 364–366.

Perham, R.N., Terry, T.D., Willis, A.E., Greenwood, J., Di Marzo Veronese, F., and Appella, E. 1995. Engineering a peptide epitope display system on filamentous bacteriophage. FEMS Microbiol. Rev. **17**: 25–31.

Pessi, A., Bianchi, E., Crameri, A., Venturini, S., Tramontano, A., and Sollazzo, M. 1993. A designed metal-binding protein with a novel fold. Nature (London), **362**: 367–369.

Peters, E.A., Schatz, P.J., Johnson, S.S., and Dower, W.J. 1994. Membrane insertion defects caused by positive charges in the early mature region of protein pIII of filamentous phage fd can be corrected by prlA suppressors. J. Bacteriol. **176**: 4296–4305.

Petersen, G., Song, D., Hugle-Dorr, B., Oldenburg, I., and Bautz, E.K.F. 1995. Mapping of linear epitopes recognized by monoclonal antibodies with gene-fragment phage display libraries. Mol. Gen. Genet. **249**: 425–431.

Petrenko, V.A., Smith, G.P., Gong, X., and Quinn, T. 1996. A library of organic landscapes on filamentous phage. Protein Eng. **9**: 797–801.

Pratt, D., Tzagoloff, H., and Erdahl, W.S. 1966. Conditional lethal mutants of the small filamentous coliphage M13. Virology, **30**: 397–410.

Pugsley, A.P. 1993. The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. **57**: 50–108.

Rampf, B., Bross, P., Vocke, T., and Rasched, I. 1991. Release of periplasmic proteins induced in *E. coli* by expression of an N-terminal proximal segment of the phage fd gene 3 protein. FEBS Lett. **280**: 27–31.

Rasched, I. and Oberer, E. 1986. Ff coliphages: structural and functional relationships. Microbiol. Rev. **50**: 401–427.

Roberts, B.L., Markland, W., Ley, A.C., Kent, R.B., White, D.W., Guterman, S.K., and Ladner R.C. 1992*a*. Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage. Proc. Natl. Acad. Sci. U.S.A. **89**: 2429–2433.

Roberts, B.L., Markland, W., Siranosian, K., Saxena, M.J., Guterman, S.K., and Ladner, R.C. 1992*b*. Protease inhibitor display M13 phage: selection of high-affinity neutrophil elastase inhibitors. Gene, **121**: 9–15.

Roberts, D., Guegler, K., and Winter, J. 1993. Antibody as a surrogate receptor in the screening of a phage display library. Gene, **128**: 67–69.

Russel, M. 1991. Filamentous phage assembly. Mol. Microbiol. **5**: 1607–1613.

Russel, M. 1995. Moving through the membrane with filamentous phages. Trends Microbiol. **3**: 223–228.

Russel, M., and Model, P. 1981. A mutation downstream from the signal peptidase cleavage site affects cleavage but not membrane insertion of phage coat protein. Proc. Natl. Acad. Sci. U.S.A. **78**: 1717–1721.

Russel, M., Wirhlow, H., Sun, T.-P., and Webster, R.E. 1988. Low-frequency infection of F^- bacteria by transducing particles of filamentous bacteriophages. J. Bacteriol. **170**: 5312–5316.

Saggio, I., and Laufer, R. 1993. Biotin binders selected from a random peptide library expressed on phage. Biochem. J. **293**: 613–616.

Saggio, I., Gloaguen, I., and Laufer, R. 1995. Functional phage display of ciliary neurotrophic factor. Gene, **152**: 35–39.

Scarselli, E., Esposito, G., and Traboni, C. 1993. Display of functional domains of the human high affinity IgE receptor on the M13 phage surface. FEBS Lett. **329**: 223–226.

Schellekens, G.A., Lasonder, E., Feijlbrief, M., Koedijk, D.G.A.M., Drijfhout, J.W., Scheffer, A.-J., Welling-Wester, S., and Welling, G.W. 1994. Identification of the core residues of the epitope of a monoclonal antibody raised against glycoprotein D of herpes simplex virus type 1 by screening of a random peptide library. Eur. J. Immunol. **24**: 3188–3193.

Scott, J.K. 1992. Discovering peptide ligands using epitope libraries. Trends Biochem. Sci. **17**: 241–245.

Scott, J.K., and Craig, L. 1994. Random peptide libraries. Curr. Opin. Biotechnol. **5**: 40–48.

Scott, J.K., and Smith, G.P. 1990. Searching for peptide ligands with an epitope library. Science (Washington, D.C.), **249**: 386–390.

Sioud, M., Dybwad, A., Jespersen, L., Suleyman, S., Natvig, J.B., and Forre, O. 1994. Characterization of naturally occurring autoantibodies against tumor necrosis factor-alpha (TNF-α): in vitro function and precise epitope mapping by phage epitope library. Clin. Exp. Immunol. **98**: 520–525.

Smith, G.P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science (Washington, D.C.), **228**: 1315–1317.

Smith, G.P. 1988. Filamentous phage assembly: morphogenetically defective mutants that do not kill the host. Virology, **167**: 156–165.

Smith, G.P. 1993. Surface display and peptide libraries. Gene, **128**: 1–2.

Smith, G.P., and Petrenko, V.A. 1997. Phage display. Chem. Rev. **97**: 391–410.

Smith, G.P., and Scott, J.K. 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol. **217**: 228–257.

Smith, G.P., Schultz, D.A., and Ladbury, J.E. 1993. A ribonuclease S-peptide antagonist discovered with a bacteriophage display library. Gene, **128**: 37–42.

Smith, J.M., and Smith, N.H. 1996. Site-specific codon bias in bacteria. Genetics, **142**: 1037–1043.

Söderlind, E., Duenas, M., and Borrebaeck, C.A.K. 1995. Chaperonins in phage display of antibody fragments. Methods Mol. Biol. **51**: 343–353.

Soumillion, P., Jespers, L., Bouchet, M., March, and Brynaert, J., Winter, G., and Fastrez, J. 1994. Selection of β-lactamase on filamentous bacteriophage by catalytic activity. J. Mol. Biol. **237**: 415–422.

Sparks, A.B., Quilliam, L.A., Thorn, J.M., Der, C.J., and Kay, B.K. 1994. Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries. J. Biol. Chem. **269**: 23 853 – 23 856.

Stephen, C.W., Helminen, P., and Lane, D.P. 1995. Characterisation of epitopes on human p53 using phage-displayed peptide libraries: insights into antibody-peptide interactions. J. Mol. Biol. **248**: 58–78.

Sternberg, N., and Hoess, R.H. 1995. Display of peptides and proteins on the surface of bacteriophage lambda. Proc. Natl. Acad. Sci. U.S.A. **92**: 1609–1613.

Summers, R.G., and Knowles, J.R. 1989. Illicit secretion of a cytoplasmic protein into the periplasm of *Escherichia coli* requires a signal peptide plus a portion of the cognate secreted protein. J. Biol. Chem. **264**: 20 074 – 20 081.

Swimmer, C., Lehar, S.M., McCafferty, J., Chiswell, D.J., Blattler, W.A., and Guild, B.C. 1992. Phage display of ricin B chain and its single binding domains: system for screening galactose-binding mutants. Proc. Natl. Acad. Sci. U.S.A. **89**: 3756–3760.

von Heijne, G. 1986. Net N–C charge imbalance may be important for signal sequence function in bacteria. J. Mol. Biol. **192**: 287–290.

von Heijne, G. 1994. Membrane proteins: from sequence to structure. Annu. Rev. Biophys. Biomol. Struct. **23**: 167–92.

Walker, G.C. 1996. The SOS response of *Escherichia coli*. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. *Edited by* F.C. Neidhardt. ASM Press, Washington, D.C. pp. 1400–1416.

Wall, J.G., and Pluckthun, A. 1995. Effects of overexpressing folding modulators on the in vivo folding of heterologous proteins in *Escherichia coli*. Curr. Opin. Biotechnol. **6**: 507–516.

Wang, C.-I., Yang, Q., and Craik, C.S. 1995*a*. Isolation of a high affinity inhibitor of urokinase-type plasminogen activator by phage display of ecotin. J. Biol. Chem. **270**: 12 250 – 12 256.

Wang, L.-F., Du Plessis, D.H., White, J.R., Hyatt, A.D., and Eaton, B.T. 1995*b*. Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5. J. Immunol. Methods, **178**: 1–12.

Wells, J.A. 1996. Hormone mimicry. Science (Washington, D.C.), **273**: 449–450.

Wells, J.A., and Lowman, H.B. 1992. Rapid evolution of peptide and protein binding properties in vitro. Curr. Opin. Biotechnol. **3**: 355–362.

Widersten, M., and Mannervik, B. 1995. Glutathione transferases with novel active sites isolated by phage display from a library of random mutants. J. Mol. Biol. **250**: 115–122.

Williams, K.A., Glibowicka, M., Li, Z., Li, H., Khan, A.R., Chen, Y.M.Y., Wang, J., Marvin, D.A., and Deber, C.M. 1995. Packing of coat protein amphipathic and transmembrane helices in filamentous bacteriophage M13: role of small residues in protein oligomerization. J. Mol. Biol. **252**: 6–14.

Wilson, D.R. 1997. Multivalent pIII phage display libraries: selected issues and applications. Ph.D. thesis, University of British Columbia, Vancouver.

Wilson, D.R., Siebers, A., and Finlay, B.B. 1997. (University of British Columbia.) Unpublished data.

Winter, G., Griffiths, A.D., Hawkins, R.E., and Hoogenboom, H.R. 1994. Making antibodies by phage display technology. Annu. Rev. Immunol. **12**: 433–455.

Wrighton, N.C., Farrell, F.X., Chang, R., Kashyap, A.K., Barbone, F.P., Mulcahy, L.S., Johnson D.L., Barrett, R.W., Jolliffe, L.K., and Dower, W.J. 1996. Small peptides as potent mimetics of the protein hormone erythropoietin. Science (Washington, D.C.), **273**: 458–464.

Yamane, K., and Mizushima, S. 1988. Introduction of basic amino acids after the signal peptide inhibits protein translocation across the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **263**: 19 690 – 19 696.

Yao, Z.-J., Kao, M.C.C., and Chung, M.C.M. 1995. Epitope identification by polyclonal antibody from phage-displayed random peptide library. J. Protein Chem. **14**: 161–166.

Yatagai, F., Halliday, J.A., and Glickman, B.W. 1991*a*. Specificity of recA441-mediated (tif-1) mutational events. Mol. Gen. Genet. **230**: 75–80.

Yatagai, F., Horsfall, M.J., and Glickman, B.W. 1991*b*. Specificity of SOS mutagenesis in native M13lacI phage. J. Bacteriol. **173**: 7996–7999.

Zacher, A.N.I., Stock, C.A., Golden, J.W.I., and Smith, G.P. 1980. A new filamentous phage cloning vector: fd-tet. Gene, **9**: 127–140.

Zhong, G., Smith, G.P., Berry, J., and Brunham, R.C. 1994. Conformational mimicry of a chlamydial neutralization epitope on filamentous phage. J. Biol. Chem. **269**: 24 183 – 24 188.